

CONSIDERED: /AG/

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

MARTINEZ *et al.*

Appl. No.: 10/669,597

Filed: September 25, 2003

For: **Polymer Conjugates with  
Decreased Antigenicity, Methods of  
Preparation and Uses Thereof**

Confirmation No.: 1312

Art Unit: 1654

Examiner: Gupta, A.

Atty. Docket: 2057.0040002/BJD/JKM

**Declaration of Merry R. Sherman Under 37 C.F.R. § 1.132**

***Mail Stop Amendment***

Commissioner for Patents

PO Box 1450

Alexandria, VA 22313-1450

Sir:

The undersigned, Merry R. Sherman, residing at 3455 Brittan Avenue, San Carlos, CA 94070, USA, declares and states as follows:

1. I am a co-inventor of above-captioned U.S. Patent Application No. 10/669,597 ("the '597 application") entitled "Polymer Conjugates with Decreased Antigenicity, Methods of Preparation and Uses Thereof," which has a filing date of September 25, 2003, which claims priority to U.S. Patent Application No. 10/317,092, filed December 12, 2002, and which claims benefit of U.S. Provisional Patent Application No. 60/414,424, filed September 30, 2002.

2. I am currently employed by Mountain View Pharmaceuticals, Inc., the assignee of the above-captioned application, where I hold the positions of President and

Atty. Dkt. No. 2057.0040002/BJD/JKM

Chief Executive Officer. My credentials are provided in the *curriculum vitae* that is attached to this declaration as Exhibit A. I received my M.A. degree in Biochemistry in 1963, and my Ph.D. degree in Biophysics in 1966, from the University of California at Berkeley. As seen from my attached *curriculum vitae*, I have published many papers related to polymer conjugation to bioactive components, including peptides and proteins. Based on my education and experience, I am an expert in the field of polymer conjugation, including PEGylation technologies.

3. I have reviewed and am familiar with the '597 application filed on September 25, 2003, the Office Action dated March 25, 2009 ("the Office Action"), issued by the U.S. Patent and Trademark Office in the present application, and the currently pending claims, filed in the Reply to Office Action with this declaration.

4. Traditionally, conjugation of water soluble polymers, such as poly(ethylene glycol) (PEG) to bioactive components such as small molecule drugs, nucleic acids, proteins, polypeptides and glycoproteins, has been used to increase the solubility, stability and persistence in the circulation of those bioactive components in animals, including humans. In all of the currently marketed bioactive components to which PEG is covalently linked, the PEG is terminated by a methoxyl group at the distal end of each linear PEG or at all distal ends of branched PEGs. This is true regardless of the nature of the chemical linkage between the polymer and the bioactive component (primarily, amide bonds, secondary amines and urethane bonds in the marketed products). The end of the polymer that is covalently attached to the bioactive component is referred to as the "proximal terminus." The opposite end of a linear polymer that is

attached at its proximal terminus to the bioactive component is referred to as the “distal terminus;” the unattached ends of a branched polymer that is attached by its proximal terminus to the bioactive component are referred to as “distal termini.”

5. Methoxyl-terminated PEGs have been used for conjugation to bioactive components as a matter of convenience in the preparation of PEGs that are made reactive at only one end (“monofunctionally activated PEGs,” *see* the '597 application at page 4, paragraph [0007]). The presently claimed invention is based, at least in part, on the recognition and demonstration by me and my co-inventors, that the distal methoxyl groups are not inert with respect to the immune system of the animal or patient to which a PEGylated bioactive component is administered. Most of the antibodies formed against the polymer component of the drug are directed against the distal methoxyl groups, and not against the backbone of the polymer, which has the general structure:



where *n* (the number of ethylene oxide units or the degree of polymerization) is about 227 for 10-kDa PEG and about 455 for 20-kDa PEG.

6. In order to overcome the immunogenicity of traditional PEG molecules, my co-inventors and I synthesized and tested PEG conjugates of numerous bioactive components made with monofunctionally activated conventional methoxyPEG (mPEG) and compared them with PEG conjugates in which all of the distal termini of the PEG are hydroxyl groups (Mountain View Pharmaceuticals, Inc. has registered the trademark, PharmaPEG®, for such hydroxyl-terminated PEGs). In each instance, the processes used for coupling mPEG and PharmaPEG to the bioactive component and for purification of

the respective conjugates were identical regardless of the PEG derivative utilized. Conjugates of several proteins conjugated with mPEG or PharmaPEG of the same molecular weight were compared with respect to biological activities in cell culture. Two immunologic parameters, antigenicity and immunogenicity, have been measured. As defined in paragraph [0003] of the '597 application, the term "antigenicity" refers to the ability of a molecule to bind to preexisting antibodies, while the term "immunogenicity" refers to the ability of the molecule to evoke an immune response *in vivo*, whether that response involves the formation of antibodies (a "humoral response") or the stimulation of cellular immune responses. As discussed in detail below, methods to make and use the full scope of the hydroxyl-terminated polyalkylene glycol conjugates (e.g., PharmaPEG conjugates) of the presently claimed invention are clearly provided in the '597 application.

7. With regard to the Office Action, at pages 3-8, the Examiner first asserts that claims 1-11, 13-15, 17-27, 30, 35, 38, 59-67, 69-71, 73-82, 85, 90, 93-96, and 101-109 are allegedly not described in the specification in such as way as to convey to one skilled in the art that the inventors, at the time the application was filed, were in possession of the claimed invention. The Examiner contends that the "specific peptide/non-peptide bioactive agents do not provide written description for all of the bioactive agents, mimetic[s], and functional antagonist[s] of the claimed invention." Office Action at page 6, lines 9-11. The Examiner has also asserted that claims 1-11, 13-15, 17-27, 30, 35, 38, 59-67, 69-71, 73-82, 85, 90, 93-96 and 101-109 allegedly contain subject matter that was not described in the '597 application in such as way as to enable one of skill in the art to make and use the invention. Specifically, the



Examiner asserts that the '597 application "does not provide any examples that demonstrate the coupling of a heterobifunctional [sic] PEG, with a free hydroxyl group, to a protein, especially GM-CSF. Such guidance is necessary because the art indicates that the strategy utilizing heterobifunctional [sic] PEG, that have [sic] a free hydroxyl group, also has its limits." Office Action at page 11, lines 6-9.

8. As discussed in detail below, a person of skill in the art would have understood that the inventors, at the time the '597 application was filed, were in full possession of the presently claimed invention and that the specification of the '597 application would have allowed such a person to make and use the presently claimed invention without unreasonable experimentation.

9. The '597 application, throughout pages 26-32, describes numerous bioactive components that can be used in the practice of the present invention. As stated at page 31, paragraph [0082], "[a]s the ordinarily skilled artisan will appreciate, any bioactive component known and readily available in the art is suitable for conjugation with monofunctional polymers having reduced antigenicity, substantially reduced antigenicity or undetectable antigenicity, according to the present invention."

10. At the time of filing the '597 application, it was well known to artisans working in the area of PEGylation technology that numerous different types of bioactive components could be coupled to PEG molecules using well-known chemical coupling reactions. For Example, as described in Fishburn, "The Pharmacology of PEGylation: Balancing PD with PK to Generate Novel Therapeutics," *Journal of Pharmaceutical Sciences* 97:4167-4183 (2008) (hereinafter "Fishburn;" Exhibit C), "[t]he first five

approved [PEGylated] products were proteins or peptides; the most recently approved, Macugen<sup>®</sup> (pegatanib), is an RNA aptamer, while studies on PEG-conjugates of small molecules such as the  $\alpha 4\beta 1$  integrin inhibitor demonstrate that the technology can extend beyond biologics and macromolecules." Fishburn at page 4168, first column, first paragraph. Greenwald *et al.*, "Effective drug delivery by PEGylated drug conjugates," *Advanced Drug Delivery Reviews* 55:217-250 (2003) (hereinafter "Greenwald;" Exhibit D) provides a review on the production of a number of PEG-bioactive component conjugates, including proteins such as  $\alpha$ -interferon, bovine adenosine deaminase and L-asparaginase, for example. Greenwald at page 219, section 2.1. Greenwald also describes the PEGylation of a number of small organic molecules, such as doxorubicin, paclitaxel (*id.* at page 220, section 2.2), camptothecin (*id.* at page 225, first column, lines 1-4) and daunorubicin (*id.* at page 231, section 3.2.1. *See also* Rodrigues *et al.*, "Acid-Sensitive Polyethylene Glycol Conjugates of Doxorubicin: Preparation, In Vitro Efficacy and Intracellular Distribution," *Bioorganic & Medicinal Chemistry* 7:2517-2524 (1999) (Exhibit E), *e.g.*, at abstract and introduction. In addition, as discussed in Greenwald, PEGylated conjugates of oligodeoxynucleotides have also been prepared (Greenwald at page 221, section 2.4), as well as PEG conjugates of antibodies and antibody fragments (*id.* at page 222, section 2.5).

11. Thus, it was well known by those in the art as of the filing date of the '597 application that PEGylated conjugates could be prepared using an array of bioactive components of widely varied molecular structure. Based on this knowledge, and the disclosure of numerous bioactive components throughout the '597 application, those of

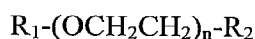
ordinary skill in the art would clearly have understood that the present inventors were in possession of the full scope of the presently claimed conjugates.

12. The conjugates of the presently claimed invention are generated by first obtaining or preparing a monofunctionally activated polyalkylene glycol molecule (for example, as described in the '597 application at pages 21-26, and Examples 5 and 6), and then conjugating the polyalkylene glycol molecule to a bioactive component (*see, e.g.*, the '597 application at pages 33-38, and Examples 1 and 7). As stated at page 21, paragraph [0060] of the '597 application, preparation of the conjugates of the present invention requires simply "substitution of such monofunctionally activated PEGs in place of monofunctionally activated mPEG" in well-known PEG conjugation methods. Thus, those of ordinary skill in the art would readily recognize that the conjugation between the hydroxyl terminated polymers of the presently claimed invention and the bioactive components, is suitably performed in the same manner as conjugations have traditionally been performed using mPEG (or other alkoxyl-terminated polymers) and the same bioactive components.

13. As described throughout the '597 application, including throughout pages 33-38, and Examples 1 and 7, conjugation of the polyalkylene glycol molecules of the present invention to the bioactive component, utilizes well-known, well-characterized coupling reactions. "The PAGs employed in the practice of the present invention, which, as indicated above, are preferably activated by reaction with a coupling group, can be attached to any of several groups that may be present on the bioactive component molecule . . . ." '597 application at page 33, paragraph [0084]. As detailed below, these

conjugation reactions (as well as others known in the art) would not have required excess experimentation to perform, and as noted above, simply required the substitution of the monofunctionally activated polymers of the present invention in place of the art-known alkoxyl-terminated polymers in the conjugation methods.

14. As described at page 4, paragraph [0007] of the '597 application, linear PEGs have the following general structure:



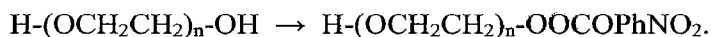
where  $R_2$  may be a hydroxyl group (or a reactive derivative thereof) and  $R_1$  may be hydrogen, as in PEG diol, a methyl group, as in monomethoxyPEG (mPEG), or another lower alkyl group, *e.g.*, *iso*-propoxyPEG or *t*-butoxyPEG. If  $R_1$  is a methyl or other alkyl group and  $R_2$  is a hydroxyl group, the unreactive polymer can be converted to a form that can react with a bioactive component at a single terminus. For example, the hydroxyl group can be reacted with *p*-nitrophenylchloroformate in a solvent of acetonitrile and pyridine to form a mono-*p*-nitrophenylcarbonate derivative of the mPEG (mPEG-NPC). The starting material and the product can be written as follows:



where Ph represents a phenyl ring. The full chemical structure of the product is shown in Figure 1A of Exhibit B attached herewith. If a sufficient quantity of *p*-nitrophenylchloroformate is added, very little of the starting material remains.

15. An analogous reaction to that described in paragraph 14 above is disclosed in Example 5, at page 60, paragraph [0147], of the '597 application. In

Example 5, PEG diol was selected as the starting polymer. As both ends of the initial starting polymer are hydroxyl groups that are able to react with the *p*-nitrophenylchloroformate, the desired monofunctionally activated product (containing a single NPC group) must be purified from the reaction mixture that could also contain unreacted PEG diol and bifunctionally activated product, i.e., DiNPC-PEG (*see* the '597 application at page 61, paragraph [0149]). Removal of the bifunctionally activated polymer is essential to avoid cross-linking of bioactive components. The purification is readily achieved by reversed phase chromatography. *Id.* The starting material and the desired product can be written as follows:



The full chemical structure of the product is shown in Figure 1B of Exhibit B.

16. Reaction of mPEG-NPC and PharmaPEG-NPC with a bioactive component containing an accessible amino group (NH<sub>2</sub>) are shown schematically in Figures 1A and 1B of Exhibit B, respectively. These reactions involving mPEG are well known in the art, as described throughout pages 33-38 of the '597 application. Conjugation of mPEG-NPC and PharmaPEG-NPC to interferon-beta (interferon-β), interleukin-2, granulocyte-macrophage colony-stimulating factor (GM-CSF), erythropoietin (EPO), uricase and human serum albumin, among others, has been performed in our laboratories. Conjugation of bioactive components to PEGs of the same molecular weight showed no differences in the reaction conditions regardless of the characteristics of the distal termini of the polymers (*i.e.*, methoxyl vs. hydroxyl groups).

17. The use of the *p*-nitrophenylcarbonate derivative of mPEG to make mPEG-uricase is described in Example 1 of the '597 application, and the synthesis of the analogous PharmaPEG conjugate of uricase is described in Example 7. More details regarding the reaction conditions of the conjugation are provided in US Patent No. 6,576,235, which was incorporated by reference and cited at page 3, paragraph [0006] of the '597 application. The use of *p*-nitrophenylcarbonate derivatives of mPEGs of various sizes to make conjugates of murine and recombinant human GM-CSF is described in Sherman *et al.*, in *Poly(ethylene glycol): Chemistry and Biological Applications*, Harris J.M., *et al.*, eds., American Chemical Society, Washington, DC, pp. 155-169 (1997); and Saifer *et al.*, *Polym. Preprints* 38:576-577 (1997), cited in the '597 application at page 3, paragraph [0005], and page 5, paragraph [0009], respectively. As noted above, substitution of the hydroxyl-terminated polymers of the presently claimed invention for the mPEGs utilized in the methods of these references, is clearly well within the level of those skilled in the art, and would not required any more than routine experimentation to perform. In unpublished experiments, our laboratory has prepared mPEG and PharmaPEG conjugates of recombinant human GM-CSF synthesized with *p*-nitrophenylcarbonate derivatives of mPEGs or PharmaPEGs of various molecular weights utilizing the same methods known in the art and described in the '597 application.

18. Another pair of analogous mPEG and PharmaPEG reagents having a single, terminal propionaldehyde as the reactive group, and either a methoxyl group or a hydroxyl group at the distal terminus, are illustrated in Figures 2A and 2B of Exhibit B, respectively. Methods of synthesis of PharmaPEG-monoaldehyde from PEG diol are

described in Example 6 of the '597 application at page 62, paragraph [0150], through page 64, paragraph [0155]. Our laboratory has synthesized conjugates of interferon- $\alpha$  (interferon- $\alpha$ ), interferon- $\beta$ , interleukin-2, granulocyte-macrophage colony-stimulating factor (GM-CSF) and erythropoietin (EPO) utilizing PEG-monoaldehydes. As described herein, conjugation of the PharmaPEG-monoaldehyde to bioactive components would require only routine experimentation, as described in the '597 application, could be carried out using well-known conjugation methods that had traditionally been used with methoxyl-terminated polymers (*see, e.g.*, pages 33-38, and Examples 1 and 7 of the '597 application).

19. Tests of the antiproliferative activities in cell culture of corresponding monoPEG (*i.e.*, a single PEG molecule) and diPEG (*i.e.*, two PEG molecules) conjugates of interferon- $\alpha$  were performed as shown in Figure 3 of Exhibit B. Using mPEG or PharmaPEG propionaldehydes, each having a molecular weight of 20 kDa, monoPEG and diPEG conjugates of interferon- $\alpha$  were synthesized and were purified using cation-exchange chromatography. The extent of purification was confirmed by analyses of the products both by size-exclusion chromatography and by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). These analyses showed only a trace of unPEGylated interferon- $\alpha$  in the preparations of monoPEG-interferon- $\alpha$  (PEG<sub>1</sub>-IFN- $\alpha$ ) and only faint traces of monoPEG-interferon- $\alpha$  and multiply PEGylated interferon- $\alpha$  in the preparations of diPEG-interferon- $\alpha$  (PEG<sub>2</sub>-IFN- $\alpha$ ). The results in Figure 3 demonstrate that the monoPEGylated conjugates made with either mPEG-aldehyde or PharmaPEG-aldehyde

have activities that are indistinguishable from each other in inhibiting the proliferation of Daudi cells (human lymphoma cells) in culture (compare the filled and open triangles). Similarly, the diPEGylated conjugates of interferon- $\alpha$  made with either mPEG-aldehyde or PharmaPEG-aldehyde have activities that are indistinguishable from each other in this assay (compare the filled and open squares). As expected, the *in vitro* activities of the monoPEGylated conjugates were lower than that of unPEGylated interferon- $\alpha$  (filled circles) and the *in vitro* activities of the diPEGylated conjugates were lower than those of the monoPEGylated conjugates.

20. When each of the four PEG conjugates of interferon- $\alpha$  was injected repeatedly into separate groups of three rabbits, the titers of antibodies formed against mPEG were consistently greater than the titers of antibodies formed against PharmaPEG. In other words, the polymer component of the mPEG conjugates was more immunogenic than the polymer component of the PharmaPEG conjugates. These unpublished results demonstrate that the nature of the distal terminal group of the polymer has no detectable effect on either the reaction conditions for synthesizing the PEG-protein conjugate or on the biological function of the polymer-protein conjugate, other than its immunogenicity.

21. As evidence that the terminal hydroxyl groups of PharmaPEG-drug conjugates do not undesirably react with protein-based or polypeptide-based drugs, it should be noted that many FDA-approved protein-based drugs are processed with, or contain in their final dosage forms, hydroxyl-terminated PEGs, either in the form of PEG diol or in the form of polyethoxylated sorbitans (often referred to as polysorbates or TWEENS<sup>®</sup>), as non-reactive carriers or stabilizers. Polysorbate 20 (also known as



TWEEN<sup>®</sup> 20) is a polysorbate surfactant of which the stability and relative non-toxicity enable its use as a detergent and emulsifier in a number of cosmetic, scientific and pharmacological applications. It is a polyoxyethylene derivative of sorbitan monolaurate and is distinguished from the other members of the TWEEN<sup>®</sup> family by the lengths of the polyoxyethylene chain and of the fatty acid ester moiety. The commercial product contains a range of chemical species. As with polysorbate 20, polysorbate 80 contains a total of 20 ethylene oxide units, but polysorbate 80 has a longer lipophilic "tail." In some cases, the PEG or polysorbate is used in processing therapeutic proteins, while in others it is added to the final product.

22. A partial listing of protein-based drugs that contain hydroxyl-terminated PEG or polysorbates as inert excipients, includes:

AVONEX<sup>®</sup> brand of interferon- $\beta$ -1a contains polysorbate 20 in the prefilled syringe (liquid) formulation (*see* Exhibit F);

CAMPATH<sup>®</sup> brand of alemtuzumab and RITUXAN<sup>®</sup> IV brand of rituximab (monoclonal antibodies) contain polysorbate 80 (*see, e.g.,* Exhibit G);

RECOMBINATE<sup>®</sup> brand of antihemophilic factor VIII contains PEG diol (*see* Exhibit H); and

KOGENATE<sup>®</sup>, REFACTO<sup>®</sup> and XYNTHA<sup>®</sup> brands of antihemophilic factor VIII contain polysorbate 80. (*see, e.g.,* Exhibit I)

23. Hydroxyl-terminated PEG and/or polysorbates are also used in processing of at least the following products:

GAMMAGARD<sup>®</sup> S/D immunoglobulin G, the production of which includes the use of both PEG diol and polysorbate (*see* Exhibit J);

FLEBOGAMMA<sup>®</sup>, VENOGLOBULIN<sup>®</sup> and POLYGAM<sup>®</sup> brands of immunoglobulin G, the production of which includes incubation of the proteins with PEG diols (*see, e.g.*, Exhibit K);

PROLASTIN<sup>®</sup> brand of alpha-1-protease inhibitor, the production of which includes exposure of the protein to PEG diol, and ARALAST<sup>®</sup> brand of alpha-1-protease inhibitor, the production of which includes exposure of the protein to both PEG diol and polysorbate 80 (*see, e.g.*, Exhibit L); and

ALPHANATE<sup>®</sup> brand of antihemophilic factor VIII, the production of which includes the use of both PEG diol and polysorbate 80 (*see* Exhibit M).

24. As evidenced by the large number of therapeutic proteins and glycoproteins that contain PEG diol or surfactants comprising polyoxyethylene derivatives in their formulation, and the use of PEG diol or such surfactants during their processing, the terminal hydroxyl groups of polyalkylene glycol molecules are clearly non-reactive toward such proteins.

25. In summary, at the time of filing the '597 application, it was well known in the art that polyalkylene glycol molecules could readily be coupled to numerous bioactive components using well-known and well-characterized chemical reactions. Disclosure of numerous bioactive components that can be used in the practice of the

present invention in the '597 application, along with the knowledge in the art of exemplary PEGylated bioactive components, provide sufficient evidence to those skilled in the art that the inventors were in possession of the full scope of the invention at the time of filing. In addition, methods for conjugating polyalkylene glycol molecules to bioactive components are disclosed in the '597 application, and were well known at the time of filing the '597 application. Thus, it would not have required extensive experimentation to prepare the conjugates of the present invention. Rather, for example, it simply required substitution of the hydroxyl-terminated polymers of the present invention, for the alkoxy-terminated polymers, in conjugation methods that were well known in the art. Therefore, it is my opinion that the present invention is both sufficiently described, and fully enabled, by the '597 application.

26. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the present patent application or any patent issued thereon.

Respectfully submitted,

Date: May 21, 2009

Merry R. Sherman  
Merry R. Sherman

977458\_1.DOC

## EXHIBIT A

**MERRY RUBIN SHERMAN, PH.D.**  
Chief Executive Officer and President  
Mountain View Pharmaceuticals, Inc.

### Education:

Wellesley College, Wellesley, MA	B.A.	1961	Chemistry
University of California, Berkeley, CA	M.A.	1963	Biochemistry
University of California, Berkeley, CA	Ph.D.	1966	Biophysics
Weizmann Institute, Rehovot, Israel	Postdoctoral	1966-1967	Polymer Science
National Institutes of Health, Bethesda, MD	Fellowships	1967-1970	Biochemistry

### Research Positions:

1970-1976 Research Associate and Associate, Department of Surgical Research,  
Sloan-Kettering Institute (SKI), New York, NY

1975-1976 Visiting Investigator, Cardiovascular Research Institute, University of California  
Medical Center, San Francisco, CA

1975-1986 Head, Endocrine Biochemistry Laboratory, SKI

1/92-8/92 Visiting Scientist, New York University Medical Center, New York, NY

1993-1995 Pharmaceutical Consultant, Mountain View, CA

1995-present President, Mountain View Pharmaceuticals, Inc.

2005-present Chief Executive Officer, Mountain View Pharmaceuticals, Inc.

**Academic Positions:** *Positions at Cornell University Graduate School of Medical Sciences (CUGSMS), New York, NY, were concurrent with those at SKI*

1971-1972 Instructor in Biochemistry, CUGSMS, New York, NY

1972-1977 Assistant Professor of Biochemistry, CUGSMS

1977-1986 Associate Professor of Biochemistry, CUGSMS

1986-1993 Professor of Biochemistry, Rutgers University, Newark, NJ

### Honors:

1957 Finalist, National Science Talent Search

1960 Elected to *Phi Beta Kappa*

1985 Outstanding Woman Scientist Award, Association for Women in Science, Metropolitan  
New York Chapter

1987 Distinguished Alumna Award, New Rochelle High School, New Rochelle, NY

### Editorial Boards and Refereeing:

1974-1978 Editorial Board, *Endocrine Research Communications*

7/78-6/81 Editorial Board, *Journal of Biological Chemistry*

7/82-6/84 Editorial Board, *Journal of Biological Chemistry*

Occasional reviews for:  
*Anal Biochem, Arch Biochem Biophys, Biochemistry, Cancer Research,  
Endocrinology, Nature, Proc Natl Acad Sci USA, Steroids*

**Special NIH Study Sections:** 2/77, 1/79, 12/82, 5/85 and 4/91

### National Committees:

9/84-6/88 Program Committee of The Endocrine Society

12/85-6/88 Board of Scientific Counselors, Natl. Institute of Child Health and Human Dev.

**Professional Memberships:** American Society of Biological Chemists, The Endocrine Society,  
American Association for Cancer Research, Society for Neuroscience, Association for  
Women in Science, American Association of Pharmaceutical Scientists.

### **Selected Publications:**

- Rubin** MM, Katchalsky A (1966). Mathematics of band centrifugation: Concentration-independent sedimentation and diffusion in shallow density gradients. Biopolymers 4:579-593.
- Rubin** MM, Changeux J-P (1966). On the nature of allosteric transitions: Implications of non-exclusive ligand binding. J Mol Biol 21:265-274.
- Changeux J-P, **Rubin** MM (1968). Allosteric interactions in aspartate transcarbamylase. III. Interpretation of experimental data in terms of the model of Monod, Wyman and Changeux. Biochemistry 7:553-561.
- Rubin** MM, Piez KA, Katchalsky A (1969). Equilibrium mechanochemistry of collagen fibers. Biochemistry 8:3628-3637.
- O'Malley BW, **Sherman** MR, Toft DO (1970). Progesterone "receptors" in the cytoplasm and nucleus of chick oviduct target tissue. Proc Natl Acad Sci USA 67:501-508.
- Sherman** MR, Corvol PG, O'Malley BW (1970). Progesterone-binding components of chick oviduct. I. Preliminary characterization of cytoplasmic components. J Biol Chem 245:6085-6096.
- O'Malley BW, Toft DO, **Sherman** MR (1971). Progesterone-binding components of chick oviduct. II. Nuclear components. J Biol Chem 246:1117-1122.
- Sherman** MR, Atienza SBP, Shansky JR, Hoffman LM (1974). Progesterone receptors of chick oviduct. Steroid-binding "subunit" formed with divalent cations. J Biol Chem 249:5351-5363.
- Sherman** MR (1975). Physical-chemical analysis of steroid hormone receptors. Methods Enzymol 36:211-234.
- Bullock LP, Bardin CW, **Sherman** MR (1978). Androgenic, antiandrogenic and synandrogenic actions of progestins: Role of steric and allosteric interactions with androgen receptors. Endocrinology 103:1768-1782.
- Sherman** MR, Pickering LA, Rollwagen FM, Miller LK (1978). Mero-receptors: Proteolytic fragments of receptors containing the steroid-binding site. Fed Proc 37:167-173.
- Sherman** MR, Tuazon FB, Miller LK (1980). Estrogen receptor cleavage and plasminogen activation by enzymes in human breast tumor cytosol. Endocrinology 106:1715-1727.
- Sherman** MR, Moran MC, Tuazon FB, Stevens Y-W (1983). Structure, dissociation, and proteolysis of mammalian steroid receptors. Multiplicity of glucocorticoid receptor forms and proteolytic enzymes in rat liver and kidney cytosols. J Biol Chem 258:10366-10377.
- Sherman** MR, Tuazon FB, Stevens Y-W, Niu E-M (1983). Oligomeric steroid receptor forms and the products of their dissociation and proteolysis. *in: Steroid Hormone Receptors: Structure and Function, Proceedings of the 57th Nobel Symposium*, Karlskoga, Sweden, 1983, (Eriksson H, Gustafsson J-Å, eds.). Amsterdam, Elsevier, pp. 3-24.
- Sherman** MR, Stevens Y-W, Tuazon FB (1984). Multiple forms and fragments of cytosolic glucocorticoid receptors from human leukemic cells and normal lymphocytes. Cancer Research 44:3783-3796.
- Sherman** MR, Stevens J (1984). Structure of mammalian steroid receptors: Evolving concepts and methodological developments. Annu Rev Physiol 46:83-105.

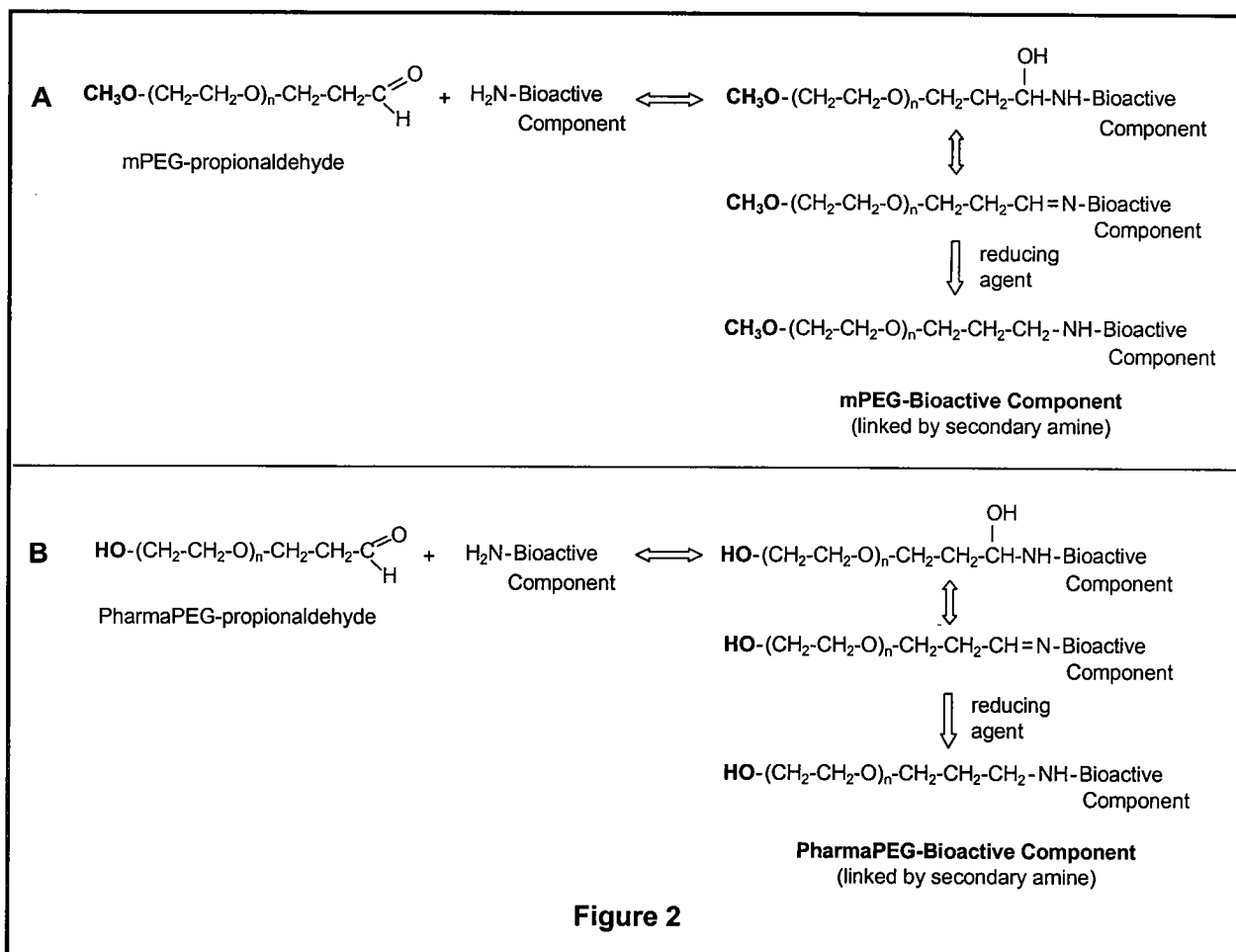
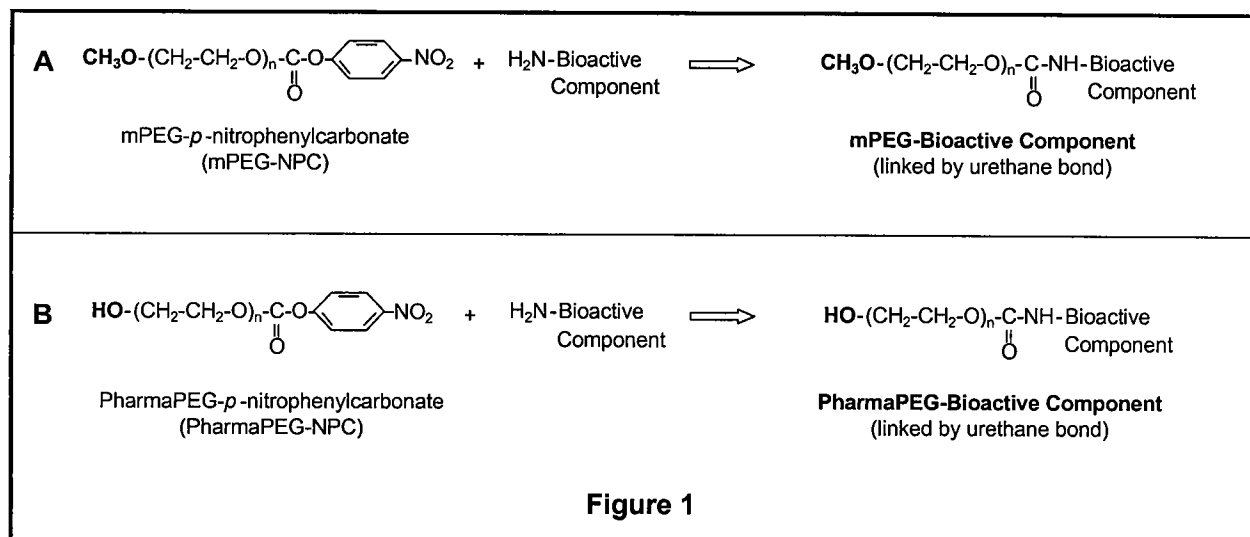
- Gorsline J, Bradlow HL, **Sherman** MR (1985). Triamcinolone acetonide 21-oic acid methyl ester: A potent local antiinflammatory steroid without detectable systemic effects. Endocrinology 116:263-273.
- Maayani S, **Sherman** MR (1990). Adenylate cyclase-linked 5-hydroxytryptamine receptors in the brain. *in: Serotonin: From Cell Biology to Pharmacology and Therapeutics*, (Paoletti R, Vanhoutte PM, Brunello N, Maggi FM, eds.). Dordrecht, The Netherlands, Kluwer Academic Publishers, pp. 39-51.
- Smith R A, Balis F M, Ott K H, Elsberry D D, **Sherman** MR, Saifer M G P (1995). Pharmacokinetics and tolerability of ventricularly administered superoxide dismutase in monkeys and preliminary clinical observations in familial ALS. J Neurol Sci 129 (Suppl): 13-18.
- Saifer M G P, Williams L D, **Sherman** MR, French J A, Kwak L W, Oppenheim J J (1997). Improved conjugation of cytokines using high molecular weight poly(ethylene glycol): PEG-GM-CSF as a prototype. Polymer Preprints 38:576-577.
- Sherman** MR, Williams L D, Saifer M G P, French J A, Kwak L W, Oppenheim J J (1997). Conjugation of high molecular weight poly(ethylene glycol) to cytokines: Granulocyte-macrophage colony-stimulating factors as model substrates. *in: Poly(ethylene glycol) Chemistry and Biological Applications. ACS Symposium Series 680*, (Harris J M, Zalipsky S, eds.). Washington, DC, American Chemical Society, pp. 155-169.
- Kelly S J, Delnomdedieu M, Oliverio M I, Williams L D, Saifer M G P, **Sherman** MR, Coffman T M, Johnson G A, Hershfield M S (2001). Diabetes insipidus in uricase-deficient mice: A model for evaluating therapy with poly(ethylene glycol)-modified uricase. J Am Soc Nephrol 12:1001-1009.
- Sherman** MR, Saifer, M G P, Perez-Ruiz, F (2008). PEG-uricase in the management of treatment-resistant gout and hyperuricemia. Adv Drug Deliv Rev 60:59-68.

#### **US and European Patents and Published Patent Applications**

- Williams L D, Hershfield M S, Kelly S J, Saifer M G P, **Sherman** MR (2003). PEG-urate oxidase conjugates and use thereof. US Patent No. 6,576,235 B1, Mountain View Pharmaceuticals, Inc., and Duke University, Jun. 10, 2003.
- Williams L D, Hershfield M S, Kelly S J, Saifer M G P, **Sherman** MR (2005). PEG-urate oxidase conjugates and use thereof. European Patent No. EP 1 100 542 B1, Mountain View Pharmaceuticals, Inc., and Duke University, Jun. 22, 2005.
- Sherman** MR, Saifer M G P, Williams L D, Hershfield M S, Kelly S J (2004). Aggregate-free urate oxidase for preparation of non-immunogenic polymer conjugates. US Patent No. 6,783,965 B1, Mountain View Pharmaceuticals, Inc., and Duke University Aug. 31, 2004.
- Sherman** MR, Saifer M G P, Williams L D, Hershfield M S, Kelly S J (2001). Aggregate-free urate oxidase for preparation of non-immunogenic polymer conjugates. PCT Patent Publication No. WO 01/59078 A2, Mountain View Pharmaceuticals, Inc., and Duke University, published Aug. 16, 2001.
- Sherman** MR, Saifer M G P, Williams L D, Hershfield M S, Kelly S J (2005). Aggregate-free protein compositions and methods of preparing same. US Patent Application No. 2005/0014240 A1, Mountain View Pharmaceuticals, Inc. and Duke University, published Jan. 20, 2005.

- Sherman MR, Martinez AL, Bhaskaran SS, Williams LD, Saifer MG P, French JA (2007).** Polymer conjugates of proteinases. US Patent 7,229,810 B2, Mountain View Pharmaceuticals, Inc., Jun. 12, 2007.
- Sherman MR, Martinez AL, Bhaskaran SS, Williams LD, Saifer MG P, French JA (2003).** Polymer conjugates of proteinases. European Patent No. EP 1 421 175 B1, Mountain View Pharmaceuticals, Inc., Nov. 19, 2008.
- Martinez AL, Sherman MR, Saifer MG P, Williams LD (2004).** Polymer conjugates with decreased antigenicity, methods of preparation and uses thereof. US Patent Application No. 2004/0062746 A1, Mountain View Pharmaceuticals, Inc., published Apr. 1, 2004.
- Martinez AL, Sherman MR, Saifer MG P, Williams LD (2004).** Polymer conjugates with decreased antigenicity, methods of preparation and uses thereof. PCT Patent Publication No. WO 2004/060617 A2, Mountain View Pharmaceuticals, Inc., published Apr. 15, 2004.
- Saifer MG P, Martinez AL, Williams LD, Sherman MR (2004).** Polymer conjugates of interferon-beta with enhanced biological potency. US Patent Application No. 2004/0126361 A1, Mountain View Pharmaceuticals, Inc., published Jul. 1, 2004.
- Saifer MG P, Martinez AL, Williams LD, Sherman MR (2004).** Polymer conjugates of interferon-beta with enhanced biological potency. PCT Patent Publication No. WO 2004/060299 A2, Mountain View Pharmaceuticals, Inc., published Jul. 22, 2004.
- Bhaskaran SS, Sherman MR, Saifer MG P, Williams LD (2004).** Polymer conjugates of cytokines, chemokines, growth factors, polypeptide hormones and antagonists thereof with preserved receptor-binding activity. US Patent Application No. 2004/0136952 A1, Mountain View Pharmaceuticals, Inc., published Jul. 15, 2004.
- Bhaskaran SS, Sherman MR, Saifer MG P, Williams LD (2004).** Polymer conjugates of cytokines, chemokines, growth factors, polypeptide hormones and antagonists thereof with preserved receptor-binding activity. PCT Patent Publication No. WO 2004/060300 A2, Mountain View Pharmaceuticals, Inc., published Jul. 22, 2004.



**Exhibit B****U.S. Patent Application No. 10/669,597****2057.0040002/BJD/JKM**

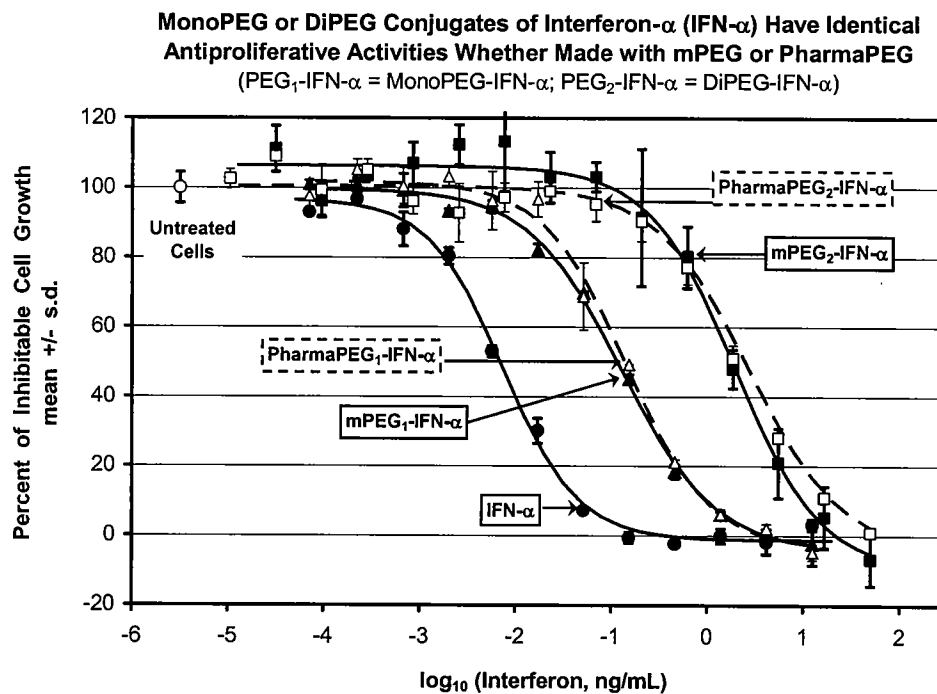


Figure 3

## REVIEW

# The Pharmacology of PEGylation: Balancing PD with PK to Generate Novel Therapeutics

C. SIMONE FISHBURN

Nektar Therapeutics, 150 Industrial Road, San Carlos, California 94070

*Received 16 September 2007; revised 9 November 2007; accepted 9 November 2007**Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jps.21278*

**ABSTRACT:** Conjugation of macromolecules to polyethylene glycol (PEG) has emerged recently as an effective strategy to alter the pharmacokinetic (PK) profiles of a variety of drugs, and thereby to improve their therapeutic potential. PEG conjugation increases retention of drugs in the circulation by protecting against enzymatic digestion, slowing filtration by the kidneys and reducing the generation of neutralizing antibodies. Often, PEGylation leads to a loss in binding affinity due to steric interference with the drug–target binding interaction. This loss in potency is offset by the longer circulating half-life of the drugs, and the resulting change in PK–PD profile has led in some cases to enabling of drugs that otherwise could not be developed, and in others to improvements in existing drugs. Thus, whereas most approaches to drug development seek to increase the activity of drugs directly, the creation of PEGylated drugs seeks to balance the pharmacodynamic (PD) and pharmacokinetic properties to produce novel therapies that will meet with both increased efficacy and greater compliance in the clinical setting. This review examines some of the PEGylated drugs developed in recent years, and highlights some of the different strategies taken to employ PEG to maximize the overall PK–PD profiles of these compounds. © 2008 Wiley-Liss, Inc. and the American Pharmacists Association *J Pharm Sci*

**Keywords:** biotechnology; conjugation; drug delivery; macromolecular drug delivery; PEGylation; pharmacokinetics/pharmacodynamics; renal clearance

## INTRODUCTION

Polyethylene glycol (PEG)-conjugated drugs first appeared on the pharmaceutical scene in 1990 with the FDA approval of Adagen® (pegademase: PEGylated adenosine deaminase), as enzyme replacement therapy for patients with severe combined immunodeficiency disease (SCID), an inherited disorder in which deficiency of adenosine deaminase causes accumulation of meta-

bolites and prevents lymphocyte maturation. The approval of Adagen followed more than a decade of research, precipitated by the first description of protein PEGylation by Abuchowski et al.<sup>1</sup> which documented the ability of PEG to prolong the half-life and reduce the immunogenicity of a conjugated protein. When applied to adenosine deaminase to produce pegademase, the technology yielded a drug that enabled twice-weekly intramuscular injections to replace multiple blood transfusions, and, by avoiding the transfusion-associated risks of viral infection and iron overload, provided both a better pharmacological profile and a considerable improvement in convenience for patients.

Correspondence to: C. Simone Fishburn (Telephone: 650-631-3482; Fax: 650-631-3150; E-mail: sfishburn@nektar.com)

*Journal of Pharmaceutical Sciences*

© 2008 Wiley-Liss, Inc. and the American Pharmacists Association



The following years saw the approval of additional PEGylated therapeutics for a number of prevalent conditions including Hepatitis C, chemotherapy-associated neutropenia and leukemia (Tab. 1). Several more PEG-conjugated compounds are currently in clinical and pre-clinical development, reflecting the emergence of this platform as a dominant strategy for enabling or improving macromolecule drugs.

The currently approved PEGylated products are all macromolecules that, between them, cross a number of therapeutic classes, including oncology, metabolic diseases and infectious diseases. The first five approved products were proteins or peptides; the most recently approved, Macugen<sup>®</sup> (pegaptanib), is an RNA aptamer, while studies on PEG-conjugates of small molecules such as the  $\alpha 4\beta 1$  integrin inhibitor demonstrate that the technology can extend beyond biologics and macromolecules.<sup>2</sup> Furthermore, clinical and pre-clinical studies have been reported which use PEG to make prodrugs for small molecules such as irinotecan,<sup>3,4</sup> doxorubicin<sup>5–7</sup> and camptothecin.<sup>8,9</sup>

Pharmacodynamic (PD) properties of a drug can be measured at the molecular level by parameters such as receptor binding affinity or enzyme activity. While PEG characteristically prolongs the plasma circulating time of a drug, a seminal PK parameter, this often comes at the expense of reduced binding affinity for the target receptor or enzyme. Thus PEG operates to alter the balance between pharmacodynamic and pharmacokinetic properties, compensating for reduction in binding affinity by extension of systemic exposure (Fig. 1). The earlier PEGylated conjugates aimed simply to

use PEG to increase systemic exposure of the drug or reduce adverse reactions, without optimizing the effect on potency. More recent approaches, however, integrate the pharmacological properties of the drug and of PEG to minimize the loss of potency while maximizing exposure.

This review will: (i) describe the pharmacological properties of PEGylated drugs, (ii) highlight two case-studies, Somavert<sup>®</sup> (pegvisomant) and PEGASYS<sup>®</sup> (peginterferon- $\alpha 2a$ ), and (iii) analyze some of the different classes of drug that can benefit from PEGylation, to demonstrate how incorporating PEG conjugation into the design of a drug is emerging as an effective strategy for improving or enabling novel therapeutics.

## PHARMACOLOGICAL PROPERTIES OF PEGYLATED DRUGS

PEG polymers are composed of repeating units of ethylene glycol, which can be produced as linear or branched chains, with functional groups at one or more termini to enable a variety of conjugation possibilities (Fig. 2). Chemical strategies for conjugating PEG to macromolecules are beyond the scope of this review and have been described in detail recently.<sup>8,10</sup> Further diversity for PEG conjugation arises from the use of either stable or hydrolyzable linkages, the latter resulting in the generation of pro-drugs. In both cases, the conjugated molecule benefits from the pharmaceutical properties of PEG, which include increased solubility, stability over a wide range

**Table 1.** FDA Approved PEGylated Drugs

Commercial Name	Drug Name	Parent Drug	PEG Size (Da)	Indication	Year of Approval
Adagen <sup>®</sup>	Pegadamas	Adenosine deaminase	5000	SCID <sup>d</sup>	1990
Oncaspar <sup>®</sup>	Pegaspargase	Asparaginase	5000	Leukaemia (ALL <sup>e</sup> , CML <sup>f</sup> )	1994
PEG-INTRON <sup>®</sup>	Peginterferon- $\alpha 2b$	IFN- $\alpha 2B$	12000	Hepatitis C	2000
PEGASYS <sup>®</sup>	Peginterferon- $\alpha 2a$	IFN- $\alpha 2A$	40000	Hepatitis C	2001
Neulasta <sup>®</sup>	Pegfilgrastim	GCSF <sup>a</sup>	20000	Neutropenia	2002
Somavert <sup>®</sup>	Pegvisomant	GH <sup>b</sup> antagonist	4–5 × 5000	Acromegaly	2003
Macugen <sup>®</sup>	Pegaptanib	Anti-VEGF <sup>c</sup> aptamer	40000	Age-related macular degeneration	2004

<sup>a</sup>GCSF, granulocyte-colony stimulating factor.

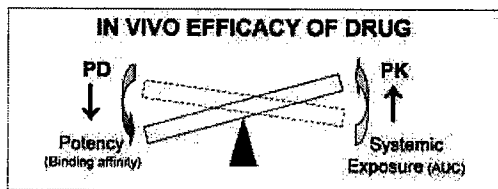
<sup>b</sup>GH, growth hormone.

<sup>c</sup>VEGF, vascular endothelial growth factor.

<sup>d</sup>SCID, severe combined immunodeficiency disease.

<sup>e</sup>ALL, acute lymphoblastic leukemia, acute lymphocytic leukemia.

<sup>f</sup>CML, chronic myeloid leukemia.18pt

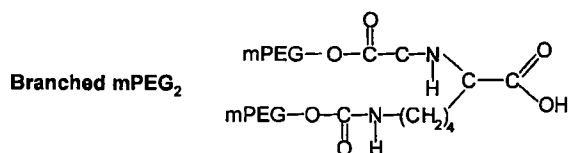


**Figure 1.** PEG alters the *in vivo* efficacy of drugs by altering the balance between their pharmacodynamic (PD) and pharmacokinetic (PK) properties. A decrease in potency caused by reduced binding affinity is compensated for by an increase in the overall systemic exposure caused by the prolonged plasma circulating time. The resulting change in the PK–PD profile provides an overall improved therapeutic efficacy that enables the generation of new drugs.

of temperature and pH, and high mobility in solution.<sup>11</sup> However, whereas for prodrugs the activity lies in the released parent molecule, stable conjugates constitute a new active species. This new molecule possesses different pharmacokinetic and pharmacodynamic properties from the parent drug although it may act at the same target receptor or enzyme. Steric hindrance created by the large PEG polymer often underlies the reduced binding affinity that results from PEG conjugation. Compensation for this is achieved through the prolonged circulation time, which, together with the reduced immunogenicity often afforded by PEG, creates an overall improved pharmacological profile that can translate not only to improved efficacy but also to reduced dosing frequency and increased patient compliance. Table 2 outlines comparative PK and PD parameters for PEGylated molecules and their unmodified parent drugs for a number of compounds. These data demonstrate how the combination of decreased activity at the target receptor or enzyme with increased plasma half-life can translate to increase efficacy in an *in vivo* animal model.

**Linear PEG-OH**      $\text{H}-(\text{OCH}_2\text{CH}_2)_n-\text{OH}$

**Linear mPEG-OH**      $\text{CH}_3-(\text{OCH}_2\text{CH}_2)_n-\text{OH}$



**Figure 2.** Structural formulae of PEG molecules. mPEG, monomethoxy PEG.

In addition, it is worth noting that PEG conjugation, by limiting diffusion across membranes, often retains drugs in the plasma compartment and results in a reduced volume of distribution.<sup>11</sup> For PEGylated prodrugs, therefore, controlled-release from the plasma compartment can be achieved by using the appropriate PEG linkers, as discussed in recent reviews.<sup>8,10</sup>

### Prolonged Circulation Time

The characteristic prolonged circulation time that PEG endows on proteins and peptides arises through two principal effects; a decrease in the rate of kidney clearance and an increase in protection from proteolytic degradation, both of which decrease the overall clearance of the drug. Since PEG polymers are highly hydrated, with two water molecules per ethylene glycol unit, their hydrodynamic radii are approximately 5- to 10-fold greater than would be predicted by their nominal molecular weight,<sup>11</sup> underlying a dramatic increase in the effective molecular size of the PEG conjugate. At lower molecular weights of PEG, clearance occurs primarily by the kidneys; above a molecular weight of approximately 20 kDa renal filtration decreases in favor of excretion by the bile, and above approximately 50 kDa, hepatobiliary clearance dominates.<sup>12,13</sup> It is worth noting that PEGylation of proteins that are normally cleared by receptor-mediated endocytosis does not generally alter the route of elimination, although some slowing of this process has been observed.<sup>14,15</sup>

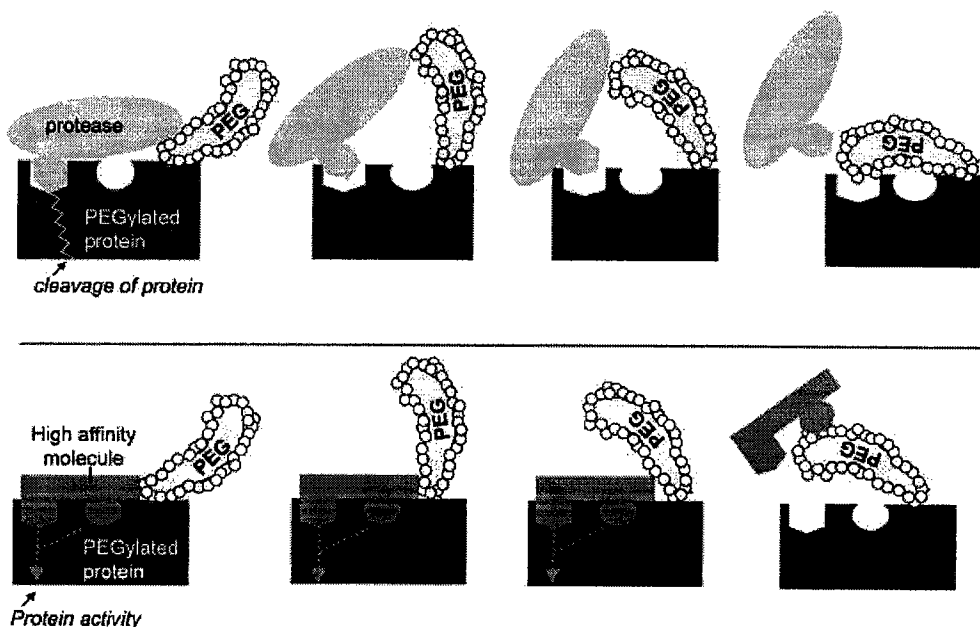
For many proteins and peptides, rapid proteolytic degradation by circulating enzymes represents one of the principal challenges in producing viable therapeutics. PEG provides protection from proteases and peptidases by impairing access for proteolytic enzymes. Despite this, the continual mobility of the PEG domain provides sufficient flexibility to enable high affinity interactions between the target receptor and the drug moiety and thus yield a biological effect. Consequently, the PEGylated drug retains efficacy while acquiring greater stability in plasma.

The most likely model to explain the protection from proteolysis involves a dynamic process in which the highly hydrated but mobile PEG moiety creates steric obstruction over the domain of the protein that serves as an enzyme substrate, thus reducing the frequency of favorable collisions. Figure 3 shows two scenarios, one involving the

**Table 2.** Comparative Pharmacokinetic and Pharmacodynamic Parameters for PEGylated Molecules and Their Parent Unmodified Drugs

Measured Parameter	PK		PD		In Vivo Efficacy		References
	Half-Life ( <i>t</i> <sub>1/2</sub> )		In Vitro Activity		In Vivo Activity		
	PK Species <sup>a</sup>	Parent Drug <i>t</i> <sub>1/2</sub> (h)	PEG-Drug <i>t</i> <sub>1/2</sub> (h)	Fold Difference	% Activity Retained <sup>b</sup>	PEG Drug Compared with Parent Drug in Animal Models	
Enzymes							
PEG-arginine deiminase	m	2.8	50	↑ × 18	48	Sustained reduction of plasma arginine to undetectable levels in mice	63
PEG-catalase	m	1	10	↑ × 10	95	10 <sup>3</sup> –10 <sup>4</sup> -fold reduction in immunogenic response in mice	1,64
PEG-methioninase	m	2	38	↑ × 19	70	12-fold longer depletion of plasma methionine levels; 10 <sup>4</sup> -fold decrease in IgG titer in mice	65,66
PEG-superoxide dismutase	r	0.01	38	↑ × 380	51	Protection from oxygen toxicity in rats and 10 <sup>3</sup> –10 <sup>4</sup> -fold reduction in immunogenic response in mice	20,64,67
PEG uricase	m	3	72	↑ × 24	100	Reduction in urate levels and protection of renal function in uricase-deficient mice. Reduced immunogenic response	44,68
Cytokines							
PEG-GCSF <sup>c</sup>	r	1.8	7.0	↑ × 3.9	41	14-fold longer effect in sustaining neutrophil count in rats	69
PEG-IFN-α2a	m	0.7	51	↑ × 73	7	Increased anti-tumor activity, elimination of neutralizing antibody response in mice	34
PEG-IFN-β1a	r	0.98	13	↑ × 13	50	2-fold increase in anti-angiogenic properties in murine tumor model	70
PEG-interleukin-6	m	0.05	48	↑ × 960	51	10-fold increase in thrombopoietic	71
PEG-TNFα <sup>d</sup>	m	0.07	0.7	↑ × 10	80	Increased tumor regression using lower doses of drug in mice	72
Polypeptide hormones							
PEG-calcitonin	r	3.31	15.4	↑ × 4.6	50	3–6-fold increase in hypocalcaemic efficacy in rats	51,73
PEG-GLP-1 <sup>e</sup>	r	0.04	0.56	↑ × 14	83	Stabilization of glycemia in diabetic mouse model and prolonged glucose-lowering effect in mice	50,52
PEG-hGH <sup>f</sup>	r	0.34	10	↑ × 29	24	2–3-fold prolongation of growth promoting activity in hypophysectomized rats	49
Antibodies							
Fab' fragments	r	0.33	9.05	↑ × 27	100	Reduced tissue distribution and greater concentration in plasma in mice. Reduced immunogenic response	58,59
Nucleic acids							
Anti-VEGF RNA aptamer	mo	24 <sup>g</sup>	94	↑ × 3.91	25	3-fold increased inhibition of VEGF-induced vascular permeability in guinea pigs	62,74

<sup>a</sup>m, mouse; r, rat; mo, monkey.<sup>b</sup>Percent parent drug activity retained in *in vitro* assay (enzyme–substrate, receptor-binding, etc.).<sup>c</sup>GCSF, granulocyte colony-stimulating factor.<sup>d</sup>TNF, tumor necrosis factor.<sup>e</sup>GLP-1, glucagon-like peptide-1.<sup>f</sup>hGH, human growth hormone.<sup>g</sup>Half-life values for parent and PEG refer to clearance from vitreous humor in the eye in monkeys.



**Figure 3.** Model for mechanism by which PEG provides proteolytic protection. Top panel: Binding of a plasma protease (light blue) to a PEGylated protein (dark blue) is impaired by the presence of the highly hydrated PEG domain (yellow, with white circles). The mobile PEG domain generates different configurations which reduce the probability of a favorable collision leading to enzyme–substrate interaction and protein cleavage. Lower panel: For the intended target binding molecule (pink) the higher affinity of the interaction drives the equilibrium to increase the probability of a productive interaction, and thus more configurations are permissive for yielding biological efficacy.

action of a proteolytic enzyme (top panel), and the other the action of a high affinity binding molecule (bottom panel). The two are distinguished by the affinity of the interaction. Thus for the lower affinity protease, accessing the necessary configuration for binding and cleavage is made considerably harder by the presence of the PEG domain. For the target binding molecule (which could be a receptor, enzyme substrate or other macromolecule), the higher affinity of the interaction increases the probability of a productive interaction, and thus biological efficacy is still achieved. Note that the presence of PEG does create steric hindrance to some degree also for this interaction, and this is reflected in the lower intrinsic activity of PEGylated molecules compared with their parent native molecule seen in Table 2.

### Reduced Immunogenicity and Toxicity

The same steric effect of the hydrated PEG chain that impairs access of proteolytic enzymes also underlies the reduced immunogenicity of PEGylated proteins. The PEG moiety minimizes the exposure of antigenic determinants, thereby

reducing or preventing the generation of neutralizing antibodies. The outcome is thus not only increased circulating half-life but also reduced toxicity of the conjugated drug. PEG itself has been approved by the FDA for use in food and cosmetics, and itself is considered essentially nontoxic.<sup>16,17</sup> Uptake of PEGylated drugs into Kupffer cells in the liver has also been reported,<sup>12</sup> and while some reports indicate that such intracellular uptake of PEGylated molecules can lead to vacuolization, no toxic consequences of this phenomenon have been observed.<sup>18–20</sup> Researchers have attempted to capitalize on this postulated cellular uptake property of PEG by using it to enhance uptake of enzymes such as catalase and superoxide dismutase into cells and deliver to their site of action.<sup>21</sup> To date, however, this has not yet proved sufficiently effective to transition to full drug development.

### TWO CASE STUDIES

The traditional paradigm for creating drugs involves synthesis of new molecular entities in

the Discovery phase which are optimized according to their activity *in vitro* and in animal models *in vivo*. Formulation approaches are commonly included later in Preclinical Development, and are employed to obtain the most favorable biopharmaceutical properties and PK profile. PEGylation provides an alternative strategy in which both the efficacy and desired PK profile are built into the design of the molecule during the Discovery phase. The first of the two case-studies describes the creation of the drug pegvisomant (Somavert®) in which PEGylation was incorporated into the molecular design of the drug at the outset, and in which PEG enabled a drug whose rapid elimination would otherwise have made it not viable as a therapeutic. The second case-study describes the development of peginterferon- $\alpha$ 2a (PEGASYS®), in which PEG conjugation was utilized on an existing drug not merely to provide a more convenient dosing regimen, but to alter the overall pharmacological efficacy of the drug as a result of its altered PK profile. Both cases highlight the potential for using PEG to alter the balance of PD and PK in a coordinated manner to provide valuable and effective new drug entities.

#### **SOMAVERT®: Pegvisomant—A Drug “Enabled” by PEGylation**

Pegvisomant was developed as a growth hormone (GH) antagonist to block the actions of hypersecreted GH in acromegaly, and represents a first-in-class therapy in the treatment of this disorder. Acromegaly is a growth disorder characterized by abnormal enlargement of the hands, feet, skull and jaw, and is caused by excessive secretion of GH, in most cases arising from a pituitary adenoma.<sup>22</sup> The hypersecreted GH activates signaling pathways that lead to overproduction of the insulin-like growth factor IGF-1 and related growth factors, whose mitogenic properties underlie the growth and metabolic clinical manifestations of acromegaly.

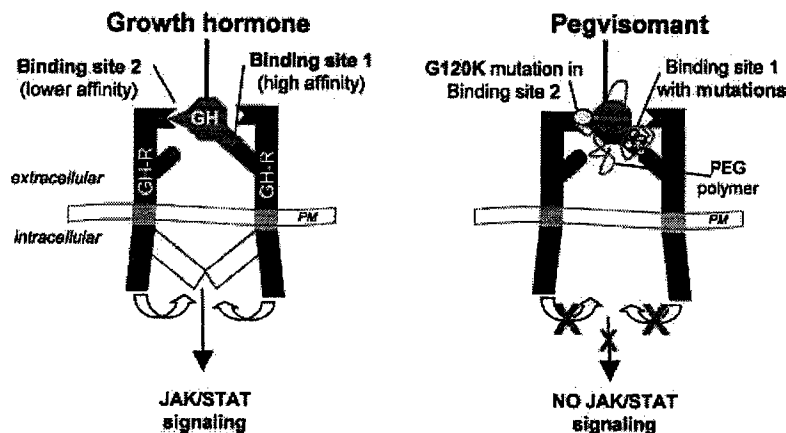
First-line therapy for acromegaly involves surgical removal of the adenoma, but this is successful in less than 60% of cases.<sup>23</sup> Alternative treatments, including radiation therapy and drug treatment with dopaminergic or somatostatin agonists show limited success and cause significant side-effects.<sup>22,24</sup> The development of pegvisomant as a GH antagonist therefore represented a breakthrough therapy and provided physicians

with a potent tool for treating this endocrine disorder.

Growth Hormone is a 22 kDa protein, secreted by somatotrophs of the anterior pituitary gland, that promotes growth and affects metabolism.<sup>25</sup> The hormone binds to a preformed dimer of the GH receptor, leading to activation of the JAK/STAT-MAP kinase signaling pathway (Fig. 4). GH contains two distinct binding sites for the receptor, termed BS1 and BS2, which display high and low affinity binding respectively. *In vitro* binding studies suggest that high affinity binding to a single receptor via the BS1 site enables interaction of the lower affinity BS2 site with a second receptor in the preformed dimer, leading to activation of the intracellular signaling cascade.<sup>26</sup>

Site-directed mutagenesis experiments demonstrated that a single point glycine to lysine mutation in the BS2 site (G120K) generates an antagonist able to bind the GH receptor with high affinity but unable to activate signal transduction.<sup>24</sup> This G120K-GH antagonist displayed a very short plasma half-life of approximately 30 min, similar to the 15 min half-life reported for the parent GH.<sup>24</sup> To prolong the plasma circulating time, 5 kDa PEG was conjugated to the antagonist using a random conjugation approach that attached PEG at free amine groups on lysine residues. This yielded a protein with a dramatically longer half-life at more than 100 h, but which lost 186-fold potency in receptor binding studies compared with GH (Tab. 3). To restore some binding potency, eight additional mutations were introduced into BS1 to increase its affinity further and compensate for the loss in affinity at BS2. Two of these, K168R and K172R, mutated lysine residues to arginine to remove PEGylation sites and thus reduce the steric hindrance generated by PEG around the binding site. The other six mutations optimized binding based on structure-function analyses of the ligand-receptor interaction.<sup>25</sup> The resulting mutated form of GH, termed B2036, behaved as a GH antagonist with similar binding affinity to that of the parent GH. PEGylation of B2036 yielded a markedly improved profile over that of the previously PEGylated antagonist. The same prolonged plasma half-life of ~100 h was produced, but the binding affinity was reduced by only 28-fold, suggesting greater retention of activity. Interestingly, binding studies using the soluble extracellular portion of the receptor demonstrated only a 4.5-fold lower affinity for PEG-B2036 than the non-PEGylated antagonist, and comparable





**Figure 4.** Model for binding of growth hormone receptor to GH (left) and pegvisomant (right). Binding of high affinity and low affinity sites on the hormone (blue shape) to sites on the extracellular domain of the GH-R in its dimer configuration causes a conformational change that activates signaling through the JAK/STAT pathway. Pegvisomant, by contrast, contains the G120K mutation (large yellow circle) and additional mutations (small yellow circles) which enable high affinity binding but cause no conformational change and hence no signal transduction, thus acting as an antagonist. GH, growth hormone; GH-R, growth hormone receptor; JAK, janus kinase; PM, plasma membrane; STAT, signal transducers and activators of transcription.

binding affinity to the parent GH,<sup>25</sup> strongly supporting the notion that the majority of the effect of PEG on binding occurs due to steric interference with the membrane-bound configuration of the receptor.

Clinical studies on pegvisomant demonstrated prolonged efficacy for up to 12 weeks,<sup>27</sup> evident as sustained suppression of total and free IGF-1, IGF Binding Protein 3 (IGFB-3) and the acid-labile subunit of IGFB-3 in patients with acromegaly (Fig. 5). Thus by combining the pharmacodynamic and pharmacokinetic properties of PEG and GH, a potent antagonist was produced that proved effective in clinical trials and which received FDA approval in 2003 as second-line therapy in treatment of acromegaly.

#### PEGASYS®: Peginterferon- $\alpha$ 2a—A Drug “Improved” by PEGylation

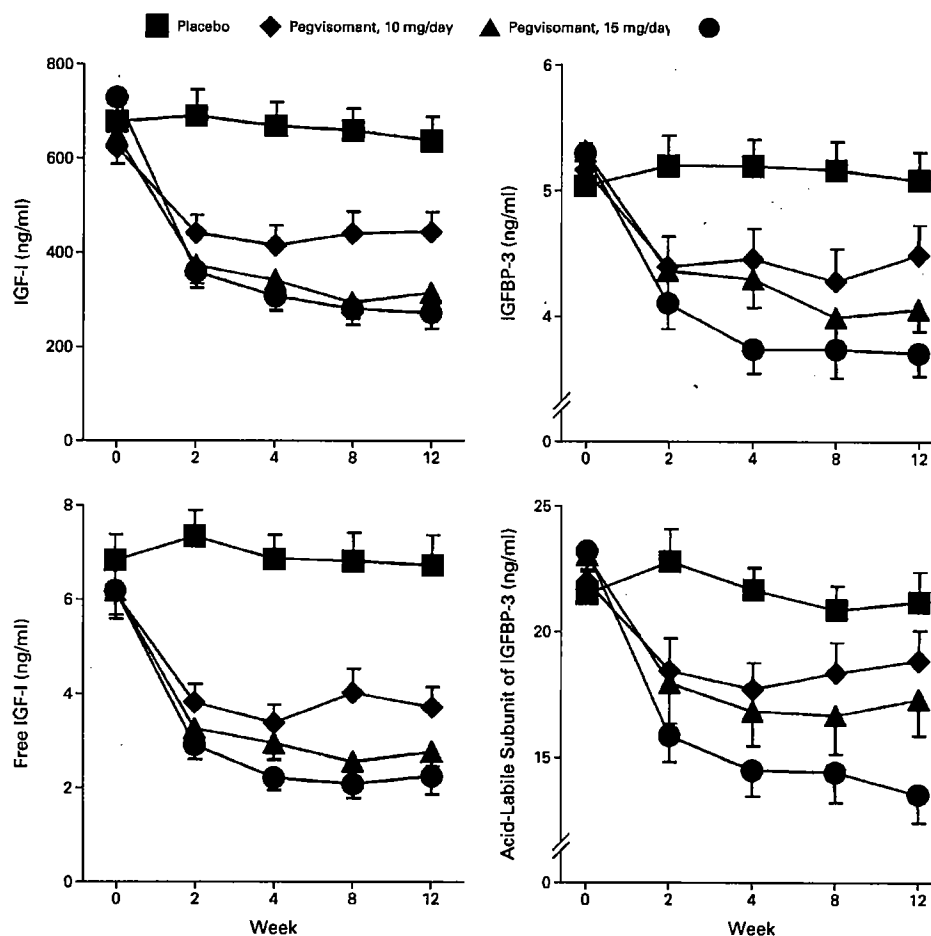
FDA approval for peginterferon- $\alpha$ 2a (PEG-IFN- $\alpha$ 2a) was granted in 2001. Since that time, this drug has obtained status as first-line therapy in chronic hepatitis C infection, and is administered either alone or in combination with the broad spectrum antiviral agent ribavirin.<sup>28</sup> Chronic hepatitis C (HCV) infection affects approximately 4 million people in the US, and represents the leading cause of hepatocellular carcinoma and liver cirrhosis.<sup>29</sup> Previous treatment of HCV infection relied on interferon- $\alpha$  (IFN- $\alpha$ ) monotherapy, and was succeeded by treatment with IFN- $\alpha$  in combination with ribavirin. The potent

**Table 3.** Relative Binding Affinities and Serum Half-Lives of GH Receptor Ligands Generated During the Discovery Process for Pegvisomant

Ligand	Binding Affinity ( $K_i$ ) at GH Receptor nM	Fold Decrease in Binding Affinity <sup>a</sup>	Serum Half-Life $t_{1/2}$ (h)	Fold Increase in Serum Half-Life ( $t_{1/2}$ ) <sup>b</sup>
GH	$3.53 \pm 0.54$	1.0	0.25	1
G120K-GH	$4.66 \pm 0.04$	1.3	0.25	1
PEG-G120K-GH	$657.1 \pm 0.71$	186	>100	>400
B2036	$3.84 \pm 0.48$	1.1	0.50	2
PEG-B2036	$99.60 \pm 6.39$	28.2	>100	>400

<sup>a</sup>Decrease in binding affinity compared with GH.

<sup>b</sup>Increase in  $t_{1/2}$  compared with GH.

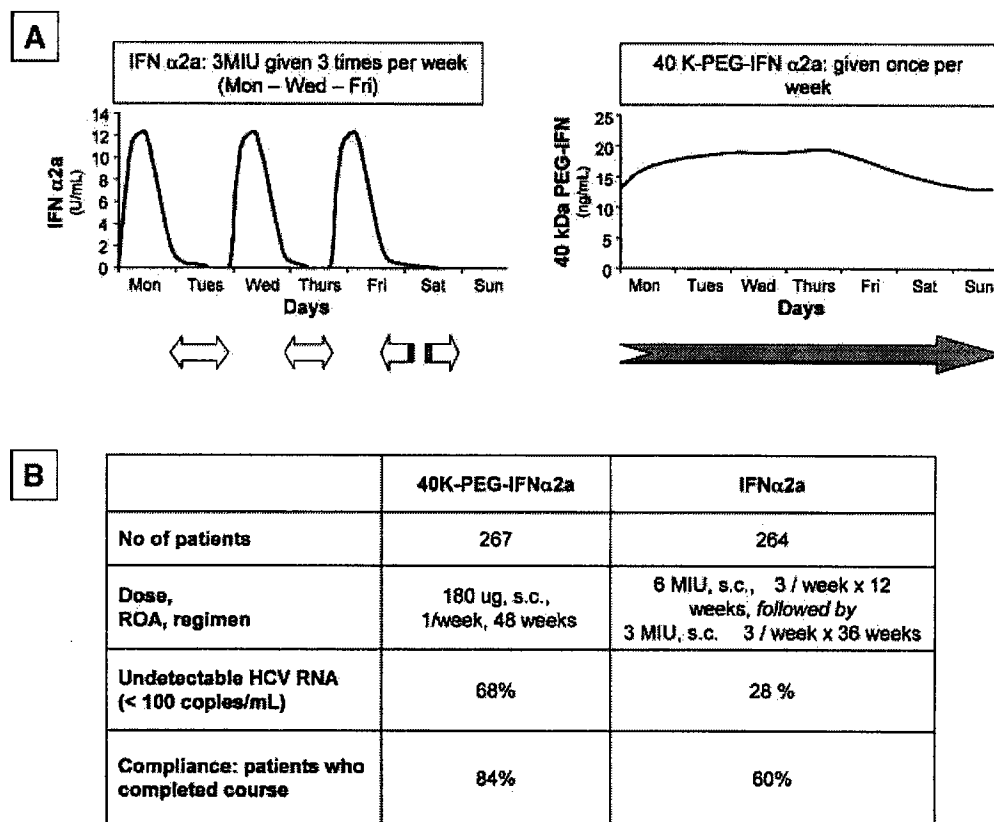


**Figure 5.** Clinical data show prolonged efficacy of pegvisomant. Following 12 weeks of treatment with pegvisomant, patients displayed significant, dose-dependent reductions in serum concentrations of total and free IGF-1, and the full and acid-labile subunits of IGF1-binding protein (IGF1BP-3) compared with placebo.

antiviral activity of IFN- $\alpha$  results from induction of interferon-stimulated genes (ISGs) via an IFN receptor-JAK/STAT mediated pathway.<sup>30</sup> Induced ISGs inhibit the translation of viral proteins and decrease the stability of the viral RNA. In addition, IFNs stimulate the innate cell-based immune response by enhancing memory T cell proliferation and natural-killer cell activation while inhibiting T cell apoptosis. The mechanism by which ribavirin augments the response to IFN is not well understood, but may involve a shift in the balance of T<sub>H</sub>1 and T<sub>H</sub>2 cells, inhibition of HCV RNA polymerase, mutagenesis of HCV RNA or GTP depletion.<sup>30</sup>

Treatment of HCV infection with unmodified interferons produces a sustained response only in a

minority of patients however, as a result of host factors such as the viral load, viral factors such as the viral genotype, and most significantly pharmacokinetic properties of the interferons themselves.<sup>28</sup> The plasma half-life of 4–6 h results in undetectable levels of IFN- $\alpha$ 2a within 24 h of a subcutaneous dose, and thus requires 3 times per week dosing by subcutaneous injection.<sup>31</sup> This produces continual fluctuations in plasma concentrations of IFN- $\alpha$ 2a, in which intervals of negligible IFN- $\alpha$ 2a creates periods of exposure to the virus, and opportunities for the virus to replicate and develop resistance mutations (Fig. 6A, left panel). The aim of PEGylation therefore, was to provide a means of obtaining a longer serum half-life, and a more consistent, “flatter” serum concentration—



**Figure 6.** The altered PK profile of PEG-IFN- $\alpha$ 2a results in a dramatic improvement in efficacy compared with unmodified IFN $\alpha$ 2a. (A) Serum concentration–time profiles for IFN $\alpha$ 2a (left panel) and PEG-IFN $\alpha$ 2a (right panel) demonstrating the ability of PEG to provide prolonged systemic exposure to the drug. Low serum concentrations of IFN $\alpha$ 2a in between doses cause periods of exposure to the virus (left panel, open arrows) which create opportunities for viral replication and development of resistance. PEG conjugation creates a prolonged continual exposure to IFN- $\alpha$ 2a (right panel, hatched arrow), during which serum concentrations are sufficiently high to minimize viral replication and mutation. (B) Clinical studies demonstrate that PEG-IFN $\alpha$ 2a, with reduced frequency of dosing, produces a significant reduction in detection of virus particles (indicated by HCV RNA copies per mL) in addition to an improvement in compliance, evident from number of patients who completed the course. HCV, hepatitis C virus; IFN, interferon; MIU, million units; ROA, route of administration; s.c., subcutaneous.

time profile with sustained levels of interferon that would generate continual anti-viral protection (Fig. 6A, right panel).

Initial attempts at PEGylation employed a 5 kDa PEG conjugated to the 19 kDa IFN- $\alpha$ 2a molecule, but generated an insufficient improvement over the unmodified IFN- $\alpha$ 2a when tested in clinical trials.<sup>11</sup> A branched 40 kDa PEG was then used to conjugate to IFN- $\alpha$ 2a, and produced a substantially different serum concentration–time profile (Fig. 6), displaying the desired PK behavior. PEGylation prolonged the serum half-life from 3.8 to 65 h, slowed the clearance by

more than 100-fold and reduced the volume of distribution fivefold.<sup>32</sup> In phase III clinical trials comparing the efficacy of treatment for 48 weeks with IFN- $\alpha$ 2a, given subcutaneously 3 times per week, with PEG-IFN- $\alpha$ 2a, subcutaneously, once per week, the response rate to the treatment was more than doubled. 68% of patients who received PEG-IFN- $\alpha$ 2a displayed undetectable levels (<100 copies HCV RNA/mL) of virus compared with only 28% of patients who received the unmodified IFN- $\alpha$ 2a. In addition, compliance was improved with the PEGylated product since the proportion of patients who

finished the course rose from 60% in the IFN- $\alpha$ 2a cohort to 84% in the PEG-IFN- $\alpha$ 2a cohort (Fig. 6B).<sup>33</sup>

Thus, by slowing renal elimination of IFN- $\alpha$ 2a and protecting from plasma proteases, PEG prolongs the circulation half-life of the molecule. Moreover, by reducing the tissue distribution and maintaining the drug primarily in the plasma where it acts on the virus, PEG increases the exposure of the virus to the drug and creates an improved therapeutic.

Interestingly, *in vitro* activity assays on the 40 kDa PEG-conjugated IFN- $\alpha$ 2a indicate that only approximately 7% of the activity of the parent molecule is retained.<sup>34</sup> By contrast, a competing product, composed of a linear 12 kDa PEG-conjugated IFN- $\alpha$ 2b (PEG-INTRON®), retains 28% of the parent compound anti-viral activity *in vitro*.<sup>35</sup> However, the improvement in systemic exposure offered by the 12 kDa PEG is relatively modest compared with that of the branched 40 kDa PEG, as it displays a half-life of 27–37 h, a 10-fold lower clearance and only a minor change in the volume of distribution.<sup>32</sup> While both forms of peginterferon are effective, these data exemplify how altering the balance between PK and PD affects the overall pharmacological profile. Thus the degree to which intrinsic activity is retained is not the sole parameter dictating the efficacy of these drugs. Equally important is the overall exposure and its relationship to the pharmacodynamics of the drug.

The current commercial formulation of PEG-IFN- $\alpha$ 2a contains a heterogeneous mixture of monoPEGylated isomers of the IFN- $\alpha$ 2a molecule. The PEGylation conjugation chemistry attaches the 40 kDa PEG molecule via amide linkages to one of nine reactive lysine residues in the IFN- $\alpha$ 2a molecule. Recent studies have separated the mixture chromatographically and examined the activity of the individual positional isomers.<sup>36,37</sup> Using VSV-infected MDBK cells and the human melanoma cell line ME15 for antiviral and antiproliferative assays respectively, Foser et al.<sup>36</sup> found that two of the isomers (K31 and K134) exhibited greater activity than the original PEG-IFN- $\alpha$ 2a, while the other seven isomers displayed less activity than the mixture. Variations were also observed between the isomers in the binding affinity for the extracellular portion of the interferon receptor IFNR2. The behavior of the different PEGylated positional isomers has been used to aid understanding of the structure-activity relationship of the IFN-receptor interac-

tion, and may enable the selection of a more potent isomer for development of a further improved PEG-interferon.

## DIFFERENT CLASSES OF DRUGS BENEFIT FROM PEGYLATION

The benefits of PEG conjugation can be leveraged differently to improve the PK-PD balance depending on the class of drug, and on its mechanism of action. This technology has been applied to multiple classes of macromolecules, including enzymes, cytokines, polypeptide hormones, antibodies and nucleic acids, as detailed in Table 4. The molecular mechanism by which PEG improves the drug is often similar within a class, resulting in similar trends for the effects of PEG on the PK and PD parameters for drugs within those classes. Therapeutic enzymes, for example, suffer relatively little loss of activity upon PEGylation whereas hormones and cytokines, which require high affinity interactions with cell surface receptors, show significant loss of binding affinity upon PEGylation (Tab. 2). For the latter, the presence of the lipid bilayer and position of the receptor allow less room for maneuver for the ligand, and the steric hindrance created by the PEG molecule thus has a greater impact on the binding affinity. For soluble enzymes which are not tethered to cellular structures, the PEG domain is sufficiently mobile and flexible to enable access of the substrate to the catalytic site, and it can more easily be conjugated at a location which has relatively little impact on the catalytic activity.

### Enzymes

Therapeutic enzymes represent a growing class of biopharmaceuticals and PEGylation has played a central role in improving or enabling several of these products.<sup>38</sup> For most enzymes examined, reduction in immunogenicity represents the principal mechanism by which PEG prolongs the circulating half life (Tab. 4). Early work on adenosine deaminase (ADA) demonstrated the ability of PEG to reduce the generation of neutralizing antibodies, extending the plasma half-life from several minutes to approximately 24 h, and led to the development of the first PEGylated enzyme therapeutic, pegademase (Adagen®), as described earlier.<sup>39</sup> Perhaps most

**Table 4.** Different Classes of Drugs Benefit from PEGylation by Differing Mechanisms of Action

Class	Molecule	PEG size (kDa)	PEGs per Molecule <sup>a</sup>	PEG Mechanism of Action	Development Status	References
Enzymes	Adenosine deaminase	5	Multiple	Proteolytic protection, reduced immunogenicity	FDA approved ( <i>Adagen</i> <sup>®</sup> )	39,40,75
	Asparaginase	5	Multiple	Reduce immunogenicity	FDA approved	42,76
	Arginine deiminase	20	22	Reduce immunogenicity	Phase I	63,77, <sup>78</sup>
	Catalase	1.9, 5	Multiple	Reduce immunogenicity, possibly facilitates cellular uptake	Preclinical	1,64,79
Cytokines	Methioninase	5	3–7	Reduce immunogenicity	Preclinical	65,80
	Superoxide dismutase	5	10–15	Reduce immunogenicity, possibly facilitates cellular uptake	Preclinical	20,64,81
	Uricase	20	Multiple	Reduce immunogenicity	Preclinical	44,45
	G-CSF	20	1	Increase size to slow renal clearance	FDA approved ( <i>Neulasta</i> <sup>®</sup> )	46
	Interferon- $\alpha$ 2a	40	1	Increase size to slow renal clearance	FDA approved ( <i>PEGASYS</i> <sup>®</sup> )	31,32
	Interferon- $\alpha$ 2b	12	1	Increase size to slow renal clearance	FDA approved ( <i>PEGINTRON</i> <sup>®</sup> )	35
	GM-CSF <sup>b</sup>	5, 10, 20	1	Increase size to slow renal clearance	Preclinical	82
	Interferon- $\beta$ 1a	20	1	Increase size to slow renal clearance	Preclinical	70,83
	Interleukin-6	5	Multiple	Increase size to slow renal clearance	Preclinical	71
	TNF $\alpha$ <sup>c</sup>	5	7–8	Increase size to slow renal clearance	Preclinical	72
Hormones	GH antagonist	5	4–5	Protection from proteases	FDA approved ( <i>Somavert</i> <sup>®</sup> )	25
	Calcitonin	2	1	Protection from proteases	Preclinical	73,84
	Growth hormone	20	1	Protection from proteases	Preclinical	48,49
	GLP-1 <sup>d</sup>	2	1	Protection from DPPIV <sup>h</sup> and other peptidases	Preclinical	50
	Fab' fragments	5, 25, 40	Multiple	Increase size to slow renal clearance	Preclinical	59
Antibodies	Anti-TNF $\alpha$ Fab'	nd <sup>e</sup>	1	Increase size to slow renal clearance	Filed for approval ( <i>Cimzia</i> <sup>®</sup> )	57
	Anti-VEGF-R2 <sup>e</sup> Fab'	nd	1	Increase size to slow renal clearance	Phase II ( <i>CDP791</i> )	56
Nucleic acids	Anti VEGF aptamer	40	1	Slow diffusion away from site of action in vitreous humor	FDA approved ( <i>Macugen</i> <sup>®</sup> )	60
	(pegaptanib)					
	Anti PDGF <sup>f</sup> aptamer	40	1	Increase size to slow renal clearance	Preclinical	85

<sup>a</sup>Number of PEG moieties attached per molecule of parent drug.<sup>b</sup>GM-CSF, granulocyte-macrophage colony-stimulating factor.<sup>c</sup>TNF $\alpha$ , tumor necrosis factor- $\alpha$ <sup>d</sup>GLP-1, glucagon-like peptide 1.<sup>e</sup>VEGF-R2, vascular endothelial growth factor receptor-2.<sup>f</sup>PDGF, Platelet-derived growth factor.<sup>h</sup>DPPIV, dipeptidylpeptidase IV.<sup>g</sup>nd, not disclosed.

interesting for this product is that it operates effectively despite not being able to reach the same cellular location as the endogenous enzyme which it replaces. While endogenous adenosine deaminase is primarily a cytosolic enzyme, the PEG component of pegademase limits its tissue distribution and retains it in the circulation, outside cells. ADA deficiency causes toxic accumulation of metabolites, in particular adenosine and 2'deoxyadenosine.<sup>40</sup> Rapid diffusion of these nucleosides across cell membranes enables them to reach the circulation, however, where pegademase can metabolize them to inosine and 2'deoxyinosine respectively, restoring the balance of metabolites necessary for recovery of immune function.<sup>41</sup>

Reduction in immunogenicity represents the primary benefit of PEGylation for a number of other enzymes under investigation for different chemotherapeutic applications, including pegasparaginase and PEG-uricase (Tab. 4). Pegasparaginase (Oncaspar®) was developed to avoid the hypersensitivity reactions which occurred in 5–20% of acute lymphoblastic leukemia (ALL) patients treated with unmodified L-asparaginase.<sup>42</sup> Treatment with L-asparaginase capitalizes on the absence of asparagine synthetase in ALL tumor cells and acts to deprive them of their external supply of the nutrient.<sup>43</sup> The ability of pegasparaginase to provide this benefit without significant immunogenicity led to the FDA approval of Oncaspar® in 1994 for ALL patients who displayed allergic reaction to the unmodified form of the drug.<sup>43</sup> Coupled with its reduced immunogenicity, the improved PK profile which enables less frequent dosing led the FDA in 2006 to expand the use of the drug for first-line treatment of patients with acute lymphoblastic leukemia (ALL) as a component of a multi-agent chemotherapy regimen.

For the enzyme uricase, immunogenicity presents a common complication and has prompted investigation of PEG-uricase for use in conditions of hyperuricemia, such as occurs during cancer chemotherapy,<sup>44</sup> where toxic levels of uric acid accumulate in the blood, or in gout.<sup>45</sup> Since humans do not express the enzyme, recombinant uricase from other species must be employed, resulting in high antigenicity due to the species difference and the nonmammalian hyperglycosylation that occurs during manufacture in yeast. By a similar mechanism to that described for proteolytic protection, illustrated in Figure 3, PEG provides a means

of shielding carbohydrate and other antigenic groups, thereby reducing the immunogenicity of the enzyme and providing the possibility of a safer therapeutic.<sup>44</sup>

## Cytokines

Cytokines are small secreted proteins involved in the regulation of immunity, inflammation and hematopoiesis, that characteristically display short plasma circulating half-lives due to their rapid clearance by the kidneys. This has complicated attempts to develop therapeutic cytokines for boosting the immune system in conditions of immunodeficiency. Since PEG conjugation to cytokines can increase their overall hydrodynamic volume, it provides a means to reduce the rate of renal filtration and extend the plasma circulating time, and has been investigated for a number of cytokines (Tab. 4).

GCSF, granulocyte-colony stimulating factor (filgrastim: Neupogen®), is a cytokine involved in stimulating production of neutrophils, whose short half-life complicates its therapeutic use for treatment of chemotherapy-induced neutropenia.<sup>46</sup> The short half-life of GCSF results from its rapid clearance, which is mediated by two pathways: renal filtration and receptor-mediated internalization. Thus, following GCSF-mediated stimulation of neutrophil generation, GCSF binds to its cognate receptors on the upregulated neutrophils and mediates its own clearance. The development of a PEGylated version of GCSF, called pegfilgrastim (Neulasta®), capitalized on the ability of PEG to affect the renal route of clearance while having a nominal effect on the neutrophil-mediated pathway. This was particularly important for this cytokine, since the negative-feedback mechanism operates to limit the number of neutrophils generated. Thus PEGylated GCSF displays slowed renal clearance, providing a significant improvement in plasma half-life, while retaining sufficient biological activity to stimulate production of neutrophils.<sup>47</sup> The newly generated neutrophils bind the circulating PEG-GCSF and remove it from the circulation by an endocytic-lysosomal pathway, thus providing a limit to the upregulation and preventing excessive formation of neutrophils which could be harmful.

Several other cytokines display benefits from the sustained plasma levels achieved by PEG conjugation. As with interferon- $\alpha$ 2a, described

previously, the altered PK profile produced by PEGylation presents opportunities not merely for convenience improvement but provides a direct benefit to the pharmacodynamics and hence efficacy of such drugs.

### Polypeptide Hormones

A number of polypeptide hormones have been investigated or developed as biotherapeutics for treatment of endocrine disorders caused by either lack of, or excessive, hormone levels. In several cases, PEGylation presents an effective strategy for providing protection from proteolytic enzymes to enable the development of viable therapeutics (Tab. 4).<sup>24,48–50</sup> As polypeptide hormones generally bind cell-surface receptors which are integral membrane proteins, the relatively bulky PEG moiety often interferes with high affinity binding to the target receptor and reduces the activity of the hormone. For this class in particular, therefore, considerable efforts have been targeted at selecting the most appropriate site on the molecule, or engineering appropriate sites, for conjugation of the PEG polymer. Early work on PEGylation of growth hormone, for example, employed a nonselective strategy that conjugated PEG to free amines on lysine residues, and demonstrated a direct relationship between number of PEG moieties and loss of binding affinity.<sup>50</sup> In this case, the increased exposure from prolonged circulation time was not sufficient to compensate for the considerable loss in activity. A more recent study, however, demonstrated that by engineering a threonine to cysteine mutation (T3C) in the GH polypeptide, a cysteine-targeted PEGylation strategy using a 20 kDa PEG could yield a monoPEGylated GH derivative that retains good binding activity and benefits from the prolonged circulation time.<sup>49</sup> Several other polypeptide hormones involved in metabolic disorders have been PEGylated to prolong their plasma circulating time, including calcitonin,<sup>51</sup> GLP-1,<sup>50,52</sup> insulin,<sup>52–54</sup> and neuropeptide Y (NPY).<sup>55</sup> In the case of calcitonin and insulin, nasal and pulmonary delivery respectively were attempted to provide a prolonged delivery system that would extend the PK–PD profile and reduce the frequency of administration.<sup>51,54</sup> In this approach, the large PEG moiety serves to slow absorption through the nasal or pulmonary epithelium, in addition to prolonging circulating time once in the systemic circulation. Here, PEG

retains the drug in the nasal cavity or lung, which effectively serve as reservoirs, and choice of the appropriate PEG size controls the rate at which the drug crosses the epithelium to reach the plasma compartment.

Incorporating the PEGylation strategy into the design of a polypeptide hormone during the Discovery phase of drug development, and considering the PK and PD characteristics early on in the process, presents promising new avenues for advancing the development of improved hormone biotherapeutics.

### Antibodies

Two products in development, Cimzia® (CDP870: PEG-anti-TNF $\alpha$  Ab) and CDP791® (PEG-anti-VEGFR-2), for treatment of rheumatoid arthritis and solid tumors respectively, are Fab' antibody fragments that employ PEG conjugation to improve the drugs' PK profile while having a minimal loss on the antibody–antigen interaction.<sup>56,57</sup> Studies aimed at determining the optimal site on IgG Fab' antibody fragments for conjugating PEG demonstrated that the hinge region cysteine residues can tolerate attachment of one or two PEG moieties, of up to total 40 kDa molecular weight, with little effect on antigen binding affinity. This is complemented by significant increases in plasma circulating times resulting from the reduced glomerular filtration by the kidneys and lower immunogenicity than the parent IgG,<sup>58,59</sup> and suggests a mechanism for successfully PEGylating antibody Fab' fragments.

These data should aid in the design of PEGylation strategies as the field of therapeutic antibody fragments continues to develop, and further antibody targets continue to be identified.

### Nucleic Acids

Pegaptanib sodium (Macugen®) represents the first approved RNA aptamer and was developed as an anti-VEGF directed therapeutic for treatment of age-related macular degeneration.<sup>60</sup> Aptamers are oligonucleotides that bind with high affinity to target molecules;<sup>61</sup> for pegaptanib the target is VEGF, a principal mediator of the angiogenesis and increased vascular permeability that lead to this form of macular degeneration. For full efficacy this drug requires intravitreal injection to enable the aptamer to access the local VEGF that

causes neovascularization. Conjugation of the 40 kDa PEG serves to slow diffusion out of the vitreous humor thus maximizing efficacy and minimizing systemic exposure. PEG conjugation causes a fourfold drop in binding affinity,<sup>62</sup> but its prolonged retention at the site of action more than compensates for this and enables sufficient VEGF inactivation to yield good clinical efficacy.

As nucleic acids provide more tools for creating drugs in the form of antisense oligonucleotides, aptamers and siRNAs, PEGylation is likely to emerge as an effective tool for improving the pharmacological profile by producing an optimal balance between the PK and PD characteristics.

## SUMMARY

PEGylation of macromolecules has advanced considerably over the last two decades, and the appearance of PEGylated drugs promises to continue to accelerate as the field of biotherapeutics expands. Understanding how PEGylation affects the pharmacology of drugs at a molecular level enables incorporation of a PEGylation strategy into drug design from the early stages in the development pathway. This optimization of the PK–PD balance early in the process may provide a more efficient approach to generating viable drug candidates, and may facilitate the creation of novel drugs from multiple classes of macromolecule.

## ACKNOWLEDGMENTS

The author thanks in particular Dr. Hema Gursahani from Nektar Therapeutics for constructive input to the manuscript, Dr. Mike Rafferty from the University of Kansas, and Dr. Mary Bossard, Dr. Andrew Clark, Dr. Jay Sisco and Dr. Ron Wolff at Nektar for their valuable comments.

## REFERENCES

1. Abuchowski A, McCoy JR, Palczuk NC, van Es T, Davis FF. 1977. Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase. *J Biol Chem* 252: 3582–3586.
2. Pepinsky RB, Lee WC, Cornebise M, Gill A, Wortham K, Chen LL, Leone DR, Giza K, Dolinski BM, Perper S, Nickerson-Nutter C, Lepage D, Chakraborty A, Whalley ET, Petter RC, Adams SP, Lobb RR, Scott DM. 2005. Design, synthesis, and analysis of a polyethylene glycol-modified (PEGylated) small molecule inhibitor of integrin  $\{\alpha\}4\{\beta\}1$  with improved pharmaceutical properties. *J Pharmacol Exp Ther* 312:742–750.
3. Antonian L, Burton K, Goodin R, Eldon MA. 2007. PEGylation governs the disposition and metabolism of irinotecan following administration of a novel PEG-Irinotecan conjugate. *European Oncology Conference 14*, ed., Barcelona, Spain.
4. Eldon MA, Antonian L, Staschen C-M, Viegas T, Singhal A. 2007. Antitumor activity and pharmacokinetics of a novel PEGylated irinotecan in irinotecan-resistant colorectal tumors implanted in mice. In *Societies FoEC*, editor. *European Cancer Conference 14*, ed., Barcelona, Spain.
5. Rodrigues PC, Beyer U, Schumacher P, Roth T, Fiebig HH, Unger C, Messori L, Orioli P, Paper DH, Mulhaupt R, Kratz F. 1999. Acid-sensitive polyethylene glycol conjugates of doxorubicin: preparation in vitro efficacy and intracellular distribution. *Bioorg Med Chem* 7:2517–2524.
6. Veronese FM, Schiavon O, Pasut G, Mendichi R, Andersson L, Tsirk A, Ford J, Wu G, Kneller S, Davies J, Duncan R. 2005. PEG-doxorubicin conjugates: Influence of polymer structure on drug release, in vitro cytotoxicity, biodistribution, and antitumor activity. *Bioconjug Chem* 16:775–784.
7. Andersson L, Davies J, Duncan R, Ferruti P, Ford J, Kneller S, Mendichi R, Pasut G, Schiavon O, Summerford C, Tirk A, Veronese FM, Vincenzi V, Wu G. 2005. Poly(ethylene glycol)-poly(ester-carbonate) block copolymers carrying PEG-peptidyl-doxorubicin pendant side chains: Synthesis and evaluation as anticancer conjugates. *Biomacromolecules* 6: 914–926.
8. Greenwald RB, Choe YH, McGuire J, Conover CD. 2003. Effective drug delivery by PEGylated drug conjugates. *Adv Drug Deliv Rev* 55:217–250.
9. Rowinsky EK, Rizzo J, Ochoa L, Takimoto CH, Forouzes B, Schwartz G, Hammond LA, Patnaik A, Kwiatek J, Goetz A, Denis L, McGuire J, Tolcher AW. 2003. A phase I and pharmacokinetic study of pegylated camptothecin as a 1-hour infusion every 3 weeks in patients with advanced solid malignancies. *J Clin Oncol* 21:148–157.
10. Roberts MJ, Bentley MD, Harris JM. 2002. Chemistry for peptide and protein PEGylation. *Adv Drug Deliv Rev* 54:459–476.
11. Harris JM, Chess RB. 2003. Effect of pegylation on pharmaceuticals. *Nat Rev Drug Discov* 2:214–221.
12. Yamaoka T, Tabata Y, Ikada Y. 1994. Distribution and tissue uptake of poly(ethylene glycol) with



- different molecular weights after intravenous administration to mice. *J Pharm Sci* 83:601–606.
13. Yamaoka T, Tabata Y, Ikada Y. 1995. Comparison of body distribution of poly(vinyl alcohol) with other water-soluble polymers after intravenous administration. *J Pharm Pharmacol* 47:479–486.
  14. Maamra M, Kopchick JJ, Strasburger CJ, Ross RJ. 2004. Pegvisomant, a growth hormone-specific antagonist, undergoes cellular internalization. *J Clin Endocrinol Metab* 89:4532–4537.
  15. Roseng L, Tolleshaug H, Berg T. 1992. Uptake, intracellular transport, and degradation of polyethylene glycol-modified asialofetuin in hepatocytes. *J Biol Chem* 267:22987–22993.
  16. Webster R, Didier E, Harris P, Siegel N, Stadler J, Tilbury L, Smith D. 2007. PEGylated proteins: Evaluation of their safety in the absence of definitive metabolism studies. *Drug Metab Dispos* 35:9–16.
  17. Pang SNJ. 1993. Final Report on the Safety Assessment of Polyethylene Glycols (PEGs) -6, -8, -32, -75, -150, -14M, -20M. *J Am Coll Toxicol* 12:429–457.
  18. Conover C, Lejeune L, Linberg R, Shum K, Shorr RG. 1996. Transitional vacuole formation following a bolus infusion of PEG-hemoglobin in the rat. *Artif Cells Blood Substit Immobil Biotechnol* 24:599–611.
  19. Harris JM, Zalipsky S. 213th Meeting of the American Chemical Society. San Francisco, CA, 1997, pp. 45–56.
  20. Tang G, White JE, Gordon RJ, Lumb PD, Tsan MF. 1993. Polyethylene glycol-conjugated superoxide dismutase protects rats against oxygen toxicity. *J Appl Physiol* 74:1425–1431.
  21. Beckman JS, Minor RL, Jr., White CW, Repine JE, Rosen GM, Freeman BA. 1988. Superoxide dismutase and catalase conjugated to polyethylene glycol increases endothelial enzyme activity and oxidant resistance. *J Biol Chem* 263:6884–6892.
  22. Melmed S. 2006. Medical progress: Acromegaly. *N Engl J Med* 355:2558–2573.
  23. Holdaway IM. 2004. Treatment of acromegaly. *Horm Res* 62:79–92.
  24. Muller AF, Kopchick JJ, Flyvbjerg A, van der Lely AJ. 2004. Clinical review 166: Growth hormone receptor antagonists. *J Clin Endocrinol Metab* 89:1503–1511.
  25. Kopchick JJ, Parkinson C, Stevens EC, Trainer PJ. 2002. Growth hormone receptor antagonists: Discovery, development, and use in patients with acromegaly. *Endocr Rev* 23:623–646.
  26. Ross RJ, Leung KC, Maamra M, Bennett W, Doyle N, Waters MJ, Ho KK. 2001. Binding and functional studies with the growth hormone receptor antagonist, B2036-PEG (pegvisomant), reveal effects of pegylation and evidence that it binds to a receptor dimer. *J Clin Endocrinol Metab* 86:1716–1723.
  27. Trainer PJ, Drake WM, Katznelson L, Freda PU, Herman-Bonert V, van der Lely AJ, Dimaraki EV, Stewart PM, Friend KE, Vance ML, Besser GM, Scarlett JA, Thorner MO, Parkinson C, Klibanski A, Powell JS, Barkan AL, Sheppard MC, Malsonado M, Rose DR, Clemmons DR, Johannsson G, Bengtsson BA, Stavrou S, Kleinberg DL, Cook DM, Phillips LS, Bidlingmaier M, Strasburger CJ, Hackett S, Zib K, Bennett WF, Davis RJ. 2000. Treatment of acromegaly with the growth hormone-receptor antagonist pegvisomant. *N Engl J Med* 342:1171–1177.
  28. Hoofnagle JH, Seeff LB. 2006. Peginterferon and ribavirin for chronic hepatitis C. *N Engl J Med* 355:2444–2451.
  29. Shepherd J, Brodin H, Cave C, Waugh N, Price A, Gabbay J. 2004. Pegylated interferon alpha-2a and -2b in combination with ribavirin in the treatment of chronic hepatitis C: A systematic review and economic evaluation. *Health Technol Assess* 8(39):1–125.
  30. Feld JJ, Hoofnagle JH. 2005. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. *Nature* 436:967–972.
  31. Grace MJ, Cutler DL, Borden RW. 2005. Pegylated IFNs for chronic hepatitis C: An update. *Expert Opin Drug Deliv* 2:219–226.
  32. Zeuzem S, Welsch C, Herrmann E. 2003. Pharmacokinetics of peginterferons. *Semin Liver Dis* 23:23–28.
  33. Zeuzem S, Feinman SV, Rasenack J, Heathcote EJ, Lai MY, Gane E, O'Grady J, Reichen J, Diago M, Lin A, Hoffman J, Brunda MJ. 2000. Peginterferon alfa-2a in patients with chronic hepatitis C. *N Engl J Med* 343:1666–1672.
  34. Bailon P, Palleroni A, Schaffer CA, Spence CL, Fung WJ, Porter JE, Ehrlich GK, Pan W, Xu ZX, Modi MW, Farid A, Berthold W, Graves M. 2001. Rational design of a potent, long-lasting form of interferon: A 40 kDa branched polyethylene glycol-conjugated interferon alpha-2a for the treatment of hepatitis C. *Bioconjug Chem* 12:195–202.
  35. Grace M, Youngster S, Gitlin G, Sydor W, Xie L, Westreich L, Jacobs S, Brassard D, Bausch J, Borden R. 2001. Structural and biologic characterization of pegylated recombinant IFN-alpha2b. *J Interferon Cytokine Res* 21:1103–1115.
  36. Foser S, Weyer K, Huber W, Certa U. 2003. Improved biological and transcriptional activity of monopegylated interferon-alpha-2a isomers. *Pharmacogenomics J* 3:312–319.
  37. Foser S, Schacher A, Weyer KA, Brugger D, Dietel E, Marti S, Schreitmuller T. 2003. Isolation, structural characterization, and antiviral activity of positional isomers of monopegylated interferon alpha-2a (PEGASYS). *Protein Expr Purif* 30:78–87.
  38. Vellard M. 2003. The enzyme as drug: Application of enzymes as pharmaceuticals. *Curr Opin Biotechnol* 14:444–450.
  39. Davis S, Abuchowski A, Park YK, Davis FF. 1981. Alteration of the circulating life and antigenic

- properties of bovine adenosine deaminase in mice by attachment of polyethylene glycol. *Clin Exp Immunol* 46:649–652.
40. Hirschhorn R. 1995. Adenosine deaminase deficiency: Molecular basis and recent developments. *Clin Immunol Immunopathol* 76:S219–S227.
  41. Hershfield MS. 1995. PEG-ADA replacement therapy for adenosine deaminase deficiency: An update after 8.5 years. *Clin Immunol Immunopathol* 76: S228–S232.
  42. Ho DH, Brown NS, Yen A, Holmes R, Keating M, Abuchowski A, Newman RA, Krakoff IH. 1986. Clinical pharmacology of polyethylene glycol-L-asparaginase. *Drug Metab Dispos* 14:349–352.
  43. Graham ML. 2003. Pegaspargase: A review of clinical studies. *Adv Drug Deliv Rev* 55:1293–1302.
  44. Bomalaski JS, Holtsberg FW, Ensor CM, Clark MA. 2002. Uricase formulated with polyethylene glycol (uricase-PEG 20): Biochemical rationale and preclinical studies. *J Rheumatol* 29:1942–1949.
  45. Sundy JS, Ganson NJ, Kelly SJ, Scarlett EL, Rehrig CD, Huang W, Hershfield MS. 2007. Pharmacokinetics and pharmacodynamics of intravenous PEGylated recombinant mammalian urate oxidase in patients with refractory gout. *Arthritis Rheum* 56:1021–1028.
  46. Molineux G. 2004. The design and development of pegfilgrastim (PEG-rmetHuG-CSF, Neulasta). *Curr Pharm Des* 10:1235–1244.
  47. Molineux G, Kinstler O, Briddell B, Hartley C, McElroy P, Kerzic P, Sutherland W, Stoney G, Kern B, Fletcher FA, Cohen A, Korach E, Ulich T, McNiece I, Lockbaum P, Miller-Messana MA, Gardner S, Hunt T, Schwab G. 1999. A new form of Filgrastim with sustained duration in vivo and enhanced ability to mobilize PBPC in both mice and humans. *Exp Hematol* 27:1724–1734.
  48. Clark R, Olson K, Fuh G, Marian M, Mortensen D, Teshima G, Chang S, Chu H, Mukku V, Canova-Davis E, Somers T, Cronin M, Winkler M, Wells JA. 1996. Long-acting growth hormones produced by conjugation with polyethylene glycol. *J Biol Chem* 271:21969–21977.
  49. Cox GN, Rosendahl MS, Chlipala EA, Smith DJ, Carlson SJ, Doherty DH. 2007. A long-acting, mono-PEGylated human growth hormone analog is a potent stimulator of weight gain and bone growth in hypophysectomized rats. *Endocrinology* 148:1590–1597.
  50. Lee SH, Lee S, Youn YS, Na DH, Chae SY, Byun Y, Lee KC. 2005. Synthesis, characterization, and pharmacokinetic studies of PEGylated glucagon-like peptide-1. *Bioconjug Chem* 16:377–382.
  51. Shin BS, Jung JH, Lee KC, Yoo SD. 2004. Nasal absorption and pharmacokinetic disposition of salmon calcitonin modified with low molecular weight polyethylene glycol. *Chem Pharm Bull (Tokyo)* 52: 957–960.
  52. Lee S, Youn YS, Lee SH, Byun Y, Lee KC. 2006. PEGylated glucagon-like peptide-1 displays preserved effects on insulin release in isolated pancreatic islets and improved biological activity in db/db mice. *Diabetologia* 49:1608–1611.
  53. Hinds KD, Kim SW. 2002. Effects of PEG conjugation on insulin properties. *Adv Drug Deliv Rev* 54: 505–530.
  54. Adis International L. 2004. Insulin Inhalation—Pfizer/Nektar Therapeutics: HMR 4006, Inhaled PEG-Insulin—Nektar, PEGylated Insulin—Nektar. *Drugs in R&D* 5:166–170.
  55. Ortiz AA, Milardo LF, DeCarr LB, Buckholz TM, Mays MR, Claus TH, Livingston JN, Mahle CD, Lumb KJ. 2007. A novel long-acting selective neuropeptide Y2 receptor polyethylene glycol-conjugated peptide agonist reduces food intake and body weight and improves glucose metabolism in rodents. *J Pharmacol Exp Ther* 323:692–700.
  56. Baka S, Clamp AR, Jayson GC. 2006. A review of the latest clinical compounds to inhibit VEGF in pathological angiogenesis. *Expert Opin Ther Targets* 10:867–876.
  57. Kaushik VV, Moots RJ. 2005. CDP-870 (certolizumab) in rheumatoid arthritis. *Expert Opin Biol Ther* 5:601–606.
  58. Chapman AP. 2002. PEGylated antibodies and antibody fragments for improved therapy: A review. *Adv Drug Deliv Rev* 54:531–545.
  59. Chapman AP, Antoniw P, Spitali M, West S, Stephens S, King DJ. 1999. Therapeutic antibody fragments with prolonged in vivo half-lives. *Nat Biotechnol* 17:780–783.
  60. Ng EW, Shima DT, Calias P, Cunningham ET, Jr., Guyer DR, Adamis AP. 2006. Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease. *Nat Rev Drug Discov* 5:123–132.
  61. Brody EN, Gold L. 2000. Aptamers as therapeutic and diagnostic agents. *J Biotechnol* 74:5–13.
  62. Ruckman J, Green LS, Beeson J, Waugh S, Gillette WL, Henninger DD, Claesson-Welsh L, Janjic N. 1998. 2'-Fluoropyrimidine RNA-based aptamers to the 165-amino acid form of vascular endothelial growth factor (VEGF165). Inhibition of receptor binding and VEGF-induced vascular permeability through interactions requiring the exon 7-encoded domain. *J Biol Chem* 273:20556–20567.
  63. Wang M, Basu A, Palm T, Hua J, Youngster S, Hwang L, Liu HC, Li X, Peng P, Zhang Y, Zhao H, Zhang Z, Longley C, Mehlig M, Borowski V, Sai P, Viswanathan M, Jang E, Petti G, Liu S, Yang K, Filpula D. 2006. Engineering an arginine catabolizing bioconjugate: Biochemical and pharmacological characterization of PEGylated derivatives of arginine deiminase from *Mycoplasma arthritidis*. *Bioconjug Chem* 17:1447–1459.

64. Nucci ML, Olejarczyk J, Abuchowski A. 1986. Immunogenicity of polyethylene glycol-modified superoxide dismutase and catalase. *J Free Radic Biol Med* 2:321–325.
65. Sun X, Yang Z, Li S, Tan Y, Zhang N, Wang X, Yagi S, Yoshioka T, Takimoto A, Mitsushima K, Suginaka A, Frenkel EP, Hoffman RM. 2003. In vivo efficacy of recombinant methioninase is enhanced by the combination of polyethylene glycol conjugation and pyridoxal 5'-phosphate supplementation. *Cancer Res* 63:8377–8383.
66. Takakura T, Takimoto A, Notsu Y, Yoshida H, Ito T, Nagatome H, Ohno M, Kobayashi Y, Yoshioka T, Inagaki K, Yagi S, Hoffman RM, Esaki N. 2006. Physicochemical and pharmacokinetic characterization of highly potent recombinant L-methionine gamma-lyase conjugated with polyethylene glycol as an antitumor agent. *Cancer Res* 66:2807–2814.
67. Pyatak PS, Abuchowski A, Davis FF. 1980. Preparation of a polyethylene glycol: Superoxide dismutase adduct, and an examination of its blood circulation life and anti-inflammatory activity. *Res Commun Chem Pathol Pharmacol* 29:113–127.
68. Kelly SJ, Delnomdedieu M, Oliverio MI, Williams LD, Saifer MG, Sherman MR, Coffman TM, Johnson GA, Hershfield MS. 2001. Diabetes insipidus in uricase-deficient mice: A model for evaluating therapy with poly(ethylene glycol)-modified uricase. *J Am Soc Nephrol* 12:1001–1009.
69. Tanaka H, Satake-Ishikawa R, Ishikawa M, Matsuki S, Asano K. 1991. Pharmacokinetics of recombinant human granulocyte colony-stimulating factor conjugated to polyethylene glycol in rats. *Cancer Res* 51:3710–3714.
70. Baker DP, Lin EY, Lin K, Pellegrini M, Petter RC, Chen LL, Arduini RM, Brickelmaier M, Wen D, Hess DM, Chen L, Grant D, Whitty A, Gill A, Lindner DJ, Pepinsky RB. 2006. N-terminally PEGylated human interferon-beta-1a with improved pharmacokinetic properties and in vivo efficacy in a melanoma angiogenesis model. *Bioconjug Chem* 17:179–188.
71. Tsutsumi Y, Kihira T, Tsunoda S, Okada N, Kaneda Y, Ohsugi Y, Miyake M, Nakagawa S, Mayumi T. 1995. Polyethylene glycol modification of interleukin-6 enhances its thrombopoietic activity. *J Control Release* 33:447–451.
72. Yamamoto Y, Tsutsumi Y, Yoshioka Y, Nishibata T, Kobayashi K, Okamoto T, Mukai Y, Shimizu T, Nakagawa S, Nagata S, Mayumi T. 2003. Site-specific PEGylation of a lysine-deficient TNF-alpha with full bioactivity. *Nat Biotechnol* 31:31.
73. Youn YS, Jung JY, Oh SH, Yoo SD, Lee KC. 2006. Improved intestinal delivery of salmon calcitonin by Lys18-amine specific PEGylation: Stability, permeability, pharmacokinetic behavior and in vivo hypocalcemic efficacy. *J Control Release* 114:334–342.
74. Drolet DW, Nelson J, Tucker CE, Zack PM, Nixon K, Bolin R, Judkins MB, Farmer JA, Wolf JL, Gill SC, Bendele RA. 2000. Pharmacokinetics and safety of an anti-vascular endothelial growth factor aptamer (NX1838) following injection into the vitreous humor of rhesus monkeys. *Pharm Res* 17: 1503–1510.
75. Hershfield MS. 1998. Adenosine deaminase deficiency: Clinical expression, molecular basis, and therapy. *Semin Hematol* 35:291–298.
76. MacEwen EG, Rosenthal R, Matus R, Viau AT, Abuchowski A. 1987. A preliminary study on the evaluation of asparaginase. Polyethylene glycol conjugate against canine malignant lymphoma. *Cancer* 59:2011–2015.
77. Ensor CM, Holtsberg FW, Bomalaski JS, Clark MA. 2002. Pegylated arginine deiminase (ADI-SS PEG20,000 mw) inhibits human melanomas and hepatocellular carcinomas in vitro and in vivo. *Cancer Res* 62:5443–5450.
78. Izzo F, Montella M, Orlando AP, Nasti G, Beneduce G, Castello G, Cremona F, Ensor CM, Holtzberg FW, Bomalaski JS, Clark MA, Curley SA, Orlando R, Scordino F, Korba BE. 2007. Pegylated arginine deiminase lowers hepatitis C viral titers and inhibits nitric oxide synthesis. *J Gastroenterol Hepatol* 22:86–91.
79. Liu TH, Beckman JS, Freeman BA, Hogan EL, Hsu CY. 1989. Polyethylene glycol-conjugated superoxide dismutase and catalase reduce ischemic brain injury. *Am J Physiol* 256:H589–H593.
80. Hoffman RM. 2003. PEG-methioninase. *Adv Exp Med Biol* 519:69–79.
81. Veronese FM, Caliceti P, Schiavon O, Sergi M. 2002. Polyethylene glycol-superoxide dismutase, a conjugate in search of exploitation. *Adv Drug Deliv Rev* 54:587–606.
82. Doherty DH, Rosendahl MS, Smith DJ, Hughes JM, Chlipala EA, Cox GN. 2005. Site-specific PEGylation of engineered cysteine analogues of recombinant human granulocyte-macrophage colony-stimulating factor. *Bioconjug Chem* 16:1291–1298.
83. Pepinsky RB, LePage DJ, Gill A, Chakraborty A, Vaidyanathan S, Green M, Baker DP, Whalley E, Hochman PS, Martin P. 2001. Improved pharmacokinetic properties of a polyethylene glycol-modified form of interferon-beta-1a with preserved in vitro bioactivity. *J Pharmacol Exp Ther* 297: 1059–1066.
84. Lee KC, Park MO, Na DH, Youn YS, Lee SD, Yoo SD, Lee HS, DeLuca PP. 2003. Intranasal delivery of PEGylated salmon calcitonins: Hypocalcemic effects in rats. *Calcif Tissue Int* 73:545–549.
85. Floege J, Ostendorf T, Janssen U, Burg M, Radeke HH, Vargeese C, Gill SC, Green LS, Janjic N. 1999. Novel approach to specific growth factor inhibition in vivo: Antagonism of platelet-derived growth factor in glomerulonephritis by aptamers. *Am J Pathol* 154:169–179.



# Effective drug delivery by PEGylated drug conjugates

Richard B. Greenwald\*, Yun H. Choe, Jeffrey McGuire, Charles D. Conover

*Enzon, Inc., 20 Kingsbridge Road, Piscataway, NJ 08854-3969, USA*

Received 10 May 2002; accepted 14 July 2002

## Abstract

The current review presents an update of drug delivery using poly(ethylene glycol) (PEG), that focuses on recent developments in both protein and organic drugs. Certainly the past 10 years has resulted in a renaissance of the field of PEG drug conjugates, initiated by the use of higher molecular weight PEGs ( $M_w > 20,000$ ), especially 40,000 which is estimated to have a plasma circulating  $t_{1/2}$  of approximately 10 h in mice. This recent resuscitation of small organic molecule delivery by high molecular weight PEG conjugates was founded on meaningful in vivo testing using established tumor models, and has led to a clinical candidate, PEG-camptothecin (PROTHECAN<sup>®</sup>), an ester based prodrug currently in phase II trials. Additional applications of high molecular weight PEG prodrug strategies to amino containing drugs are presented: similar tripartate systems based on lower  $M_w$  PEG and their use with proteins is expounded on. The modification of a benzyl elimination tripartate prodrug specific for mercaptans is presented, and its successful application to 6-mercaptopurine giving a water soluble formulation is discussed. Recent novel PEG oligonucleotides and immunoconjugates are also covered. Clinical results of FDA approved PEGylated proteins are also presented.

© 2002 Elsevier Science B.V. All rights reserved.

**Keywords:** Poly (ethylene glycol); Prodrugs; Tripartate prodrugs; Camptothecin; Paclitaxel; Daunorubicin, Ara-C; Amphotericin B; 6-Mercaptopurine; Doxorubicin; Lysozyme

## Contents

1. Introduction .....	218
2. Drug delivery using permanent PEGylation .....	219
2.1. Permanent PEGylation of proteins .....	219
2.2. Permanent PEGylation of small organic molecules .....	220
2.3. Permanent PEGylation of peptides .....	220
2.4. PEG oligodeoxynucleotides (ODN) .....	221
2.5. PEG antibodies and fragments .....	222
3. Releasable PEGylation–PEG prodrugs .....	222
3.1. Bipartate PEG prodrugs .....	223
3.1.1. Low $M_w$ ( $LM_w$ ) PEG esters .....	223
3.1.2. High $M_w$ ( $HM_w$ ) PEG esters and carbonates .....	223
3.1.3. Enhancement of activity by employment of spacer groups .....	227
3.1.4. Hydrazone–acid activated PEG–drug conjugates .....	230

\*Corresponding author. Tel.: +1-732-980-4924; fax: +1-732-885-2950.

E-mail address: [richard.greenwald@enzon.com](mailto:richard.greenwald@enzon.com) (R.B. Greenwald).

3.2. Tripartate PEG conjugate.....	230
3.2.1. Benzyl elimination (BE) system.....	231
3.2.2. Trimethyl Lock Lactonization (TML).....	234
3.2.3. Tripartate delivery of bipartate prodrugs (tetrapartate system).....	237
3.3. PEG prodrugs of water-soluble small molecule, Ara-C.....	237
3.4. Proprotein conjugates using releasable PEG (rPEG).....	239
3.5. PEG-peptide conjugates: PEG-DPDPE.....	244
3.6. Other applications of PEG prodrugs.....	244
4. Hybrid rPEGylation of protein—a strategy.....	244
5. PEG prodrug targeting.....	244
6. Conclusion.....	245
Acknowledgements.....	245
References.....	245

## 1. Introduction

Poly (ethylene glycol) (PEG, Fig. 1) is a unique polyether diol, usually manufactured by the aqueous anionic polymerization of ethylene oxide, although other polymerization initiators can be employed. Initiation of ethylene oxide polymerization using anhydrous alkanols such as methanol or derivatives including methoxyethoxy ethanol results in a mono-alkyl capped poly (ethylene glycol) such as methoxy PEG (mPEG). The polymerization reactions can be modulated and a variety of molecular weights (1000–50,000) can be obtained with low polydispersities ( $M_w/M_n$ ),  $<1.05$ . These polymers are amphiphilic and dissolve in organic solvents as well as in water; they are also non-toxic and eliminated by a combination of renal and hepatic pathways thus making them ideal to employ in pharmaceutical applications. In fact the FDA has approved PEG for Human intravenous (i.v.), oral, and dermal applications. PEG has the lowest level of protein or cellular absorption of any known polymer [1]. These properties have been exploited in numerous ways including grafting PEG to surfaces to prevent deposition of proteinaceous material. This can be further extrapo-

lated to prevention of bacterial surface growth as well as conjugation to proteins which prevents recognition by the immune system. These unusual properties of PEG may in part be due to the highly hydrated polyether backbone which is capable of acceptor hydrogen bonding, and which gives rise to a large exclusion volume (in solution PEG of a given molecular weight ( $M_w$ ) is much larger than a protein of comparable  $M_w$  [2]). A host of other applications of PEG have been reported [3] making PEG and its derivatives one of the most utilized polymers in the vast array of macromolecular organic compounds available. In a rather surprising report by Roy [4] it was suggested that PEG itself induces apoptosis in HT-29 colorectal cancer cells. While the mechanism of this finding was not accounted for, a range of PEG  $M_w$ s from 400 to 35,000 was examined and it was determined that  $M_w$  8000 produced the greatest efficacy. Finally, another excellent use of PEG is as a soluble polymer-support in organic synthesis [5].

In this review, we will mainly focus on the use of PEG in drug delivery of organic molecules and briefly touch on protein delivery. Styrene-maleic anhydride neocarzinostatin (SMANCS) copolymer [6], hydroxypropyl methacrylamide (HPMA) copolymer [7], dextran [8,9], polyglutamic acid [10], and polyaspartic acid [11] are but a few of other polymeric systems that have been employed to accomplish delivery in analogous ways. However, PEG conjugation has been most frequently approved for human use with therapeutic proteins, and offers the unique advantage of being a telechelic or semitelechelic polymer [12] and thus loaded quite predictably with organic species. Currently, new strategies have been developed which allow the delivery of several

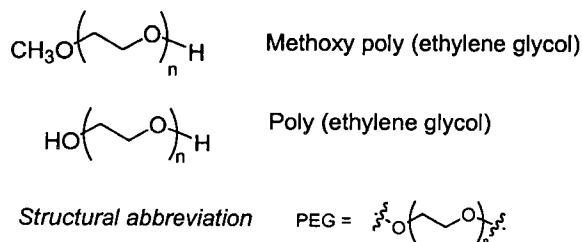


Fig. 1. Structures of poly (ethylene glycol).

active classes of small molecule PEG-conjugates, whose loading can be accurately determined by UV methods. Anticancer agents have particularly benefited from this technology, but on-going investigations of other potent medicinal agents should soon extend these applications. Most of these recent approaches will be discussed in this review. When appropriate, applications to larger proteins will be presented as well.

## 2. Drug delivery using permanent PEGylation

### 2.1. Permanent PEGylation of proteins

The potential value of proteins as therapeutics has been recognized for years. Unfortunately, many therapeutic human proteins still suffer from short circulating  $t_{1/2}$  and low stability, and therefore require the use of high doses to maintain therapeutic efficacy. This in turn increases the chance for the development of an adverse immune response [13]. Abuchowski, Davis and co-workers first described a method for the covalent attachment of mPEG to proteins in 1977 [14], since termed *PEGylation*. The PEG conjugate obtained had the properties of a significantly increased circulating  $t_{1/2}$ , reduced immunogenicity and antigenicity, and retention of a large portion of the bioactivity. It has been postulated that these effects are due to a shell of mPEG molecules around the protein that sterically hinders the reaction with immune cells and protects the adduct from proteolytic inactivation. The conjugation of mPEG with the protein (mono substituted PEG is preferred here to prevent cross-linking) is accomplished mainly by reaction with available amino groups (both  $\alpha$  and  $\epsilon$ ), although other reactive sites such as histidine and cysteine can be employed. In order to accomplish coupling, PEG must first be activated. This can be done in a number of ways and generally employs active esters, active carbonates, maleimides (thiol reactive) [15], and thiazolidine thiones [16].

The utility of PEGylation (*supra* *vida*) has been translated into drug delivery of non-human proteins. Thus, PEGylated (amide bond, PEG  $M_w$  5000) bovine adenosine deaminase (ADAGEN<sup>®</sup>) has been

successfully commercialized by Enzon, Inc. in replacement therapy (FDA approved in 1990) to treat severe combined immunodeficiency disease [17]. Bovine adenosine deaminase, with a  $M_w$  of 40,000, is cleared rapidly from the plasma of experimental animals [18]. The PEG-modified enzyme contains multiple strands of PEG-5000 per molecule of enzyme, and has a  $t_{1/2}$  about 6.4 times that of unmodified enzyme in rats [13]. Clinical pharmacokinetic and pharmacodynamic (depletion of adenosine metabolites) results support once weekly subcutaneous dosing in patients with ADA-deficient severe combined immunodeficiency disease (ADA-SCID). Long-term improvement of the immune status of these patients with chronic ADAGEN treatment has been reported [17].

Enzon also utilized PEGylation for effective drug delivery of a second biologically active protein, L-asparaginase. This PEGylated construct (ONCASPAR<sup>®</sup> approved by FDA in 1994, amide bond, PEG  $M_w$  5000) was shown to be as effective as the native drug in treating patients with acute lymphoblastic leukemia, but with a much lower degree of immunogenicity than the native. The native L-asparaginase enzyme from the bacterium *E. coli* has a  $M_w$  of about 135,000 and has been used in leukemia treatment since the 1960s [19]. PEG-L-asparaginase contains multiple strands of PEG ( $M_w$  5000), conferring about three times increased circulating  $t_{1/2}$ . Importantly, the PEG conjugated enzyme can be used successfully in many patients who have developed hypersensitivity to the highly immunogenic unmodified bacterial enzyme [20].

The year 2000 saw the first regulatory approval of PEG modified  $\alpha$ -interferon ( $\alpha$ -IFN), PEG-INTRON<sup>®</sup> (PEG IFN  $\alpha$ -2b), for treatment of hepatitis C. PEG-INTRON contains a single strand of PEG ( $M_w$  12,000) per IFN ( $M_w$  19,000). The PEG-12,000 is first activated as the succinimidyl carbonate derivative (SC-PEG), which is then reacted with the protein. The resulting product is a mixture of positional isomers where the PEG strand is attached to any one of several target amino acids with some permanent as well as some non-permanent bonds [21–23]. The plasma circulating  $t_{1/2}$  of PEG-INTRON is about eight times that of native IFN  $\alpha$ -2b, allowing for weekly subcutaneous dosing of the conjugated protein. The continuous presence of

circulating IFN activity allows for increased anti-viral efficacy compared to thrice weekly dosing of the unmodified protein. PEG-INTRON is also under study for a variety of other clinical indications including various cancers, multiple sclerosis and HIV/AIDS.

Early work on proteins often utilized PEG of  $M_w$  5000. However, several groups soon found that fewer strands of PEG of higher  $M_w$  could be employed, and led to higher specific activity and longer circulating  $t_{1/2}$  for the conjugates [24]. A recent report describes another PEGylated version of  $\alpha$ -IFN (PEGASYS, amide bond,  $M_w$  40,000) that is currently in clinical trials [25]. The unmodified IFN- $\alpha$  2a differs from that in PEG-INTRON by only one amino acid residue, and the two native proteins have similar biological activities. PEGASYS contains a single strand of a branched PEG [26,27] of total  $M_w$  40,000 per IFN- $\alpha$  molecule. The large branched PEG resulted in prolonged profiles for absorption from the subcutaneous injection site and for clearance from plasma. The anti-viral activities of PEG-INTRON and PEGASYS seem to be similar in the context of combination therapy of hepatitis C with ribavirin, although direct comparative studies have not been reported.

A PEG-conjugated form of granulocyte-colony stimulating factor (G-CSF) recently received marketing approval from the U.S. FDA [28]. This conjugate, known as NEULASTA™ or PEG-filgrastim, contains a single strand of PEG-20,000 covalently bound to the N-terminal amino group of the methionyl residue of G-CSF [29]. The single strand of PEG increases the  $M_w$  from 19,000 for G-CSF to 39,000 for PEG-filgrastim, thus reducing renal clearance. G-CSF is used to reduce the duration and severity of neutropenia that commonly occurs after cytotoxic cancer chemotherapy. Clinical trials established that a single 100  $\mu$ g/kg dose of NEULASTA, administered the day after cytotoxic chemotherapy, was equivalent to up to 14 daily doses of 5  $\mu$ g/kg of unPEGylated G-CSF in terms of days of severe neutropenia, depth of ANC (absolute neutrophil count) nadir, and time to ANC recovery after nadir.

Clinical results have also been reported for several other PEG-conjugated drugs, including IL-2 [30], growth hormone antagonist [31], hemoglobin [32] and a staphylokinase mutein [33]. In each case the conjugated protein has an extended circulating  $t_{1/2}$  in

plasma, an effect that is most striking for proteins of  $M_w$  less than about 40,000, and which would otherwise be cleared rapidly through renal filtration.

Thus, it can be concluded that permanent PEGylation of biologically active proteins greatly increases aqueous solubility, decreases immunogenicity, and still permits binding of the proteins to receptors. In addition, due to the increase in the overall  $M_w$ , the conjugate has a longer circulating  $t_{1/2}$  leading to a greater bioavailability, and thus results in less frequent dosing. The field of PEG-protein conjugates has been reviewed often and extensively [1,13,34,35].

## 2.2. Permanent PEGylation of small organic molecules

In contrast to the successful application of permanently bonded PEG to proteins (macromolecules) for drug delivery, only a few small organic molecule anticancer agents have been conjugated to PEG with permanent bonds, and those did not lead to clinically superior compounds. For example, stable amide derivatives of doxorubicin (Dox) and amphetamine [36] were prepared, but only in vitro data was reported. Permanent PEGylation of paclitaxel was also reported as a means of solubilizing and delivering this very potent anticancer drug [37]. Water soluble mPEG 5000 paclitaxel-7-carbamates were synthesized (Fig. 2) but  $10^3$  less active than the native drug in vitro and non-toxic in mice [38]. This may be attributed to two reasons: the large PEG substituent blocks activity at the target cells or the PEGylated paclitaxel does not reach the cells in sufficient concentration to produce meaningful results. This latter point will be brought out in Section 3.1.2. It was concluded from this study that a PEG *prodrug* approach to anticancer drug delivery would offer a more effective route for enhancement of therapeutic index.

## 2.3. Permanent PEGylation of peptides

The area of peptide PEGylation, while still in its infancy, has generated much interest in the drug delivery community. While it is beyond the scope of this review to cover the entire literature, the follow-

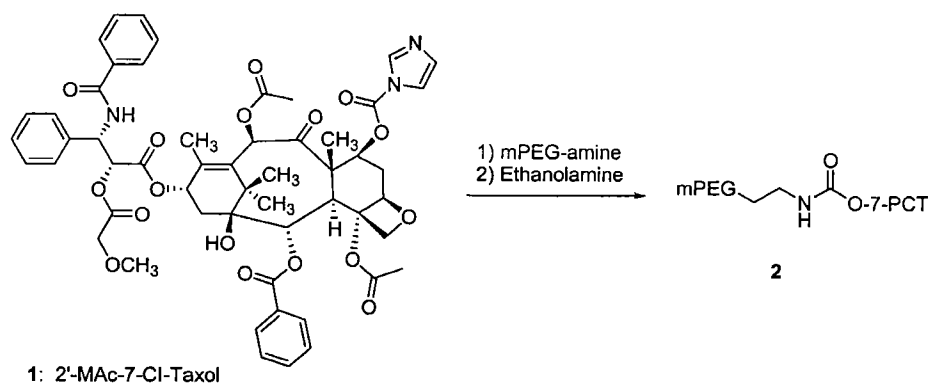


Fig. 2. PEG-carbamate-paclitaxel.

ing examples illustrate this type of PEG application to these low  $M_w$  biopolymers.

Hirudin is a naturally occurring anticoagulant polypeptide found in the leech, *Hirudo medicinalis*, and consists of 65 amino acids with an approximate  $M_w$  of 7000. Recombinant hirudin (r-hirudin) has proved to be an effective antithrombotic agent and is currently in clinical trials. It has been determined that r-hirudin is rapidly eliminated from circulation with a terminal  $t_{1/2}$  of 50–100 min, thus requiring continuous infusion or multiple daily injections to maintain therapeutic levels. Modification of r-hirudin with two strands of PEG-5000 via urethane bonds affords PEG-hirudin that exhibits a significantly prolonged  $t_{1/2}$  resulting in enhanced antithrombotic activity and no observable immunogenicity [39].

Salmon calcitonin (sCT) is a therapeutic polypeptide hormone consisting of 32 amino acids ( $M_w$  3432). It possesses a disulfide bridge between the 1 and 7 positions and a C-terminal proline amide residue. PEG modified sCT was prepared using SC-PEG as the linking agent to produce mono and di-PEGylated species [40]. Both derivatives showed slightly enhanced pharmacokinetics and lower renal excretion compared to the unmodified peptide. The authors concluded that PEGylated sCTs may have therapeutic potential.

PEGylated derivatives of peptides based on a heterobifunctional PEG linker offer the potential of longer-lived targeted peptides: a bifunctional PEG hybrid of fibronectin-related peptides has been reported [41]. The peptides, Arg-Gly-Asp (RGD) and Glu-Ile-Leu-Asp-Val (EILDV), reported as active

fragments of fibronectin (a cell adhesion protein), were conjugated with aaPEG (amino acid type PEG,  $M_w$  10,000). The hybrid, RGD-aaPEG-EILDV, was prepared by a combination of solid-phase and solution methods. Antiadhesive activity of the peptides was not lost by its hybrid formation with the large aaPEG molecule. A mixture of RGD (0.43 mmol) and EILDV (0.43 mmol) did not demonstrate an antiadhesive effect, but the hybrid containing 0.43 mmol of each peptide did exhibit this effect.

#### 2.4. PEG oligodeoxynucleotides (ODN)

The delivery of ODNs—especially as antisense agents—has been a topic of great interest in the scientific community during the past 10 years and has been reviewed [42]. The use of ODNs as possible drugs for human therapy is often prevented by their low in vivo stability and inability to reach effective concentrations at their cellular targets. The use of PEG conjugated to ODNs to assist in their solubilization and to aid their cellular uptake has been considered for some time, but the field is still in its infancy [43,44]. PEG conjugates of ODNs have mainly been introduced at the 3'- and 5'-terminus of the ODN chain, and are therefore phosphate ester derivatives [45]. Even the use of U-PEG (branched PEG) [26] has been reported for this purpose [46–49]. Recently, it was shown that a 13-mer AG motif ODN covalently linked to PEG ( $M_w$  9000) exhibited uptake and biological properties that were superior to those of the non-PEGylated sequence [50]. In addi-



tion, the PEG-ODN derivative was more resistant against S1 and fetal bovine serum nucleases than the free ODN, and less inclined to self-associate into multistrand structures in solution.

### 2.5. PEG antibodies and fragments

A natural extension of PEGylated proteins is to modify antibodies, both single chain (SCA) and monoclonal (mAb), in order to pass on the same benefits that were discussed earlier; viz. greater solubility and longer circulating life in vivo. Recently, PEGylated SCA with different  $M_w$  sizes and different styles (linear and branched) of PEG were reported [51]. The results showed that PEGylation with longer PEG was more effective than multiple PEGylation with short PEG for serum  $t_{1/2}$  extension and site specific PEG-hydrazide conjugation to carboxylic acid moieties achieved significant activity retention in bioconjugates. A clear demonstration that PEG modification of antibodies could be a benefit was reported [52]. PEG (40,000) was conjugated to the mAbs, N12 and L26, specific to the ErbB2 (HER2) oncoprotein. These antibodies suppress the growth of tumors over-expressing ErbB2. mPEG-maleimide conjugated to the sulfhydryl moieties at the hinge region impaired antibody binding to N87 cells and did not enhance the tumor inhibitory effect of the mAb. Branched PEG activated as an NHS ester and conjugated through the protein amino groups was used next with either the whole mAb or its Fab' fragment. When these derivatives were tested against N87 cells in vitro, the binding activity and antitumor cytotoxic effects of the conjugates were preserved. The PEG conjugates also demonstrated activity in vivo. On the other hand, an increase in the circulating serum  $t_{1/2}$  of the F(ab')<sub>2</sub> form of a humanized anti-interleukin IL-8 was the objective of a Genentech group study [53]. To achieve a significant increase in the hydrodynamic size of the fragment with minimal loss of bioactivity, as many as four linear mPEGs of  $M_w$  20,000 or two branched PEGs of  $M_w$  40,000 were conjugated to the  $\epsilon$ -amino groups of lysine amino acid residues as well as the  $\alpha$ -amino groups using NHS activated esters. Addition of two branched  $M_w$  40,000 PEGs increased the serum  $t_{1/2}$  to 48 h as compared to 8.5 h of the parent F(ab')<sub>2</sub>.

A very novel and potentially useful application of PEG mAb to an ADEPT (antibody directed enzyme prodrug therapy) system was disclosed by Roffler and coworkers [54]. In this work, the F(ab')<sub>2</sub> fragment of the anti-TAG-72 antibody, B72.3, was covalently linked to  $\beta$ -glucuronidase ( $\beta$ G) that was modified with mPEG 5000. The conjugate, B72.3- $\beta$ G-PEG, localized to a peak concentration in LS174T xenografts within 48 h of injection, but enzyme activity persisted in plasma such that addition of a *potentially* toxic prodrug had to be delayed for at least 4 days to avoid systemic activation. Conjugate levels in the tumor decreased to 36% of peak levels by this time. Intravenous administration of AGP3, an IgM mAb against mPEG, accelerated clearance of the conjugate from serum and increased the tumor/blood ratio of B72.3- $\beta$ G-PEG from 3.9 to 29.6 without significantly decreasing the accumulation of the conjugate in the tumor. Thus, treatment of B72.3- $\beta$ G-PEG followed 48 h later with AGP3 and a glucuronide prodrug of *p*-hydroxyaniline mustard significantly delayed tumor growth with a minimum of toxicity.

### 3. Releasable PEGylation–PEG prodrugs

Prodrug design comprises an area of drug research that is concerned with the optimization of drug delivery. A prodrug is a biologically inactive derivative of a parent drug molecule that usually requires an enzymatic transformation within the body in order to release the active drug, and has improved delivery properties over the parent molecule [55–58]. Too rapid breakdown of the prodrug can lead to spiking of the parent drug and possible toxicity, while too slow a release rate will compromise the drug's efficacy. In the case of PEG conjugates it is clear that the solubility of the prodrug will almost always exceed that of the original drug, usually overcoming any existing aqueous insolubility and thus increasing the possibility of more effective drug delivery. If the conjugate persists in the body for a period of time, either in the compartment of administration or moving from one compartment to another, it can potentially release drug as long as it stays in the body. Of course, the rate of drug release will be dependent on the nature of the polymer-drug linkage

and linkages could theoretically be chosen so that either pH or enzymatic degradation mediates prolonged drug release. Thus the stability of the drug conjugate linkage and its potential for controlled degradation determines the effectiveness of the prodrug. A general rule is that if the conjugate is designed as a circulatory depot, the drug must be liberated according to a prescribed regime without immediate total dissociation following administration. Similarly, if the conjugate is meant to reach a particular extracellular or intracellular target, the linkage must be sufficiently stable to maintain its chemical integrity until the destination is reached. The increased circulating  $t_{1/2}$  resulting from PEG conjugation can also benefit water-soluble drugs where rapid elimination is often problematic. In the case of cancerous solid tumors, it will be seen that site-specific delivery is also possible when employing a PEG prodrug strategy.

### 3.1. Bipartate PEG prodrugs

The most often employed prodrugs generally are based on hydrolyzable or enzymatically cleaveable bonds such as esters, carbonates, carbamates, and hydrazones. In special cases certain amides can be broken down in plasma as well as in the lysosomal compartment by peptidases or cathepsins. Cleavage of the particular bond employed frees the drug and the inert cohort of the combination. Thus, this type of prodrug is referred to as bipartate, or consisting of two parts. Of the bipartate prodrugs, esters are the most ubiquitous in the literature, no doubt since they are often the easiest to synthesize. Prodrugs of this sort can be designed from either an alcohol with an acid parent drug, or an alcohol parent drug with an acid. The rate of breakdown of the ester is generally more easily controlled for an alcohol drug by modification of the associated acid structure.

#### 3.1.1. Low $M_w$ ( $LM_w$ ) PEG esters

Paclitaxel, an extremely effective anticancer diterpene species, has a water solubility  $<0.01$  mg/mL [59], and possesses a 2° alcohol in the 2'-position that is necessary for activity. Esters employing PEG, which can be considered as an electron-withdrawing substituent (alkoxy) in the  $\alpha$ -position of the acid portion, are especially effective linking groups in the design of the prodrug since they aid in

the rapid hydrolysis of the ester carbonyl bond, and are thus able to release paclitaxel in a continuous and effective manner. Highly water soluble 2'-PEG 5000 esters of paclitaxel were synthesized in 1994 (Fig. 3) [60], and shown to function as prodrugs i.e. breakdown occurred in a predictable fashion: in vitro the  $t_{1/2}$  of in PBS buffer at pH 7.4 was 5.5 h, while in rat plasma a more rapid breakdown was observed, with a  $t_{1/2}$  of about 1 h. Cell tissue culture employing P338/0 leukemia cells with **4** gave  $IC_{50}$  values which were comparable to TAXOL® (cremophor EL® formulated paclitaxel). It was therefore surprising that no acute toxicity or efficacy was exhibited in mice when treated i.p. with **4** at a dose of 50 mg/kg since TAXOL at this dose was profoundly toxic [61]. This example clearly illustrates the necessity for in vivo testing to verify in vitro cytotoxicity results.

#### 3.1.2. High $M_w$ ( $HM_w$ ) PEG esters and carbonates

Traditional prodrugs are generally designed to be cleaved efficiently and rapidly ( $t_{1/2} < 20$  min) by enzymatically mediated processes resulting in an accelerated rate of conversion of the inert form to the biologically active parent [55]. Thus, the PK of the parent drug is only minimally affected by prodrug modification. However, in addition to this approach, an alternative solution to the problem of prodrug efficacy would be to extend the circulating lifetime of the water soluble modification. By increasing the circulating life of the prodrug in plasma relative to its rate of hydrolysis, equivalent or greater potency should result with a gradual controlled release of the drug as long as therapeutic levels can be reached and maintained without causing toxicity. This statement can be summarized as:

$$ED_{50} \text{ Prodrug} \leq ED_{50} \text{ Drug} \quad (1)$$

when  $t_{1/2} \text{ Circ.} > t_{1/2} \text{ Hydrolysis}$

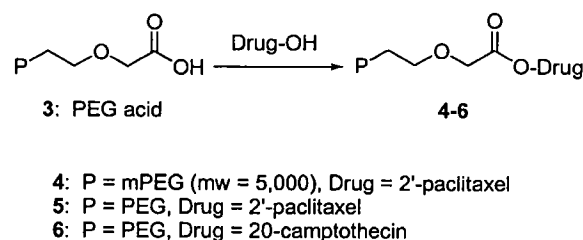


Fig. 3. PEG ester drug.

Consequently, this strongly suggests that if a means of prolonging the circulating  $t_{1/2}$  of the prodrug can be accomplished, then linking moieties of greater stability, which have previously not been considered useful, can be used in constructing prodrugs. An informative report [62] on the effect of  $M_w$  of HPMA copolymers on body distribution and rate of excretion identified a  $M_w$  threshold limiting glomerular filtration to  $M_w$  45,000; below this limit the  $t_{1/2}$  of the polymer was quite short, e.g.  $t_{1/2}$  for a  $M_w$  12,000 copolymer was reported to be only 3 min. A detailed study by Yamaoka et al. [63] found that in mice the renal clearance of i.v. injected PEG decreased with an increase in  $M_w$ , with the most dramatic change occurring at  $M_w$  30,000. The  $t_{1/2}$  of PEG circulating in blood also showed a concomitant and dramatic increase. For instance,  $t_{1/2}$  for PEG went from approximately 18 min to 16.5 h as the  $M_w$  increased from 6000 to 50,000. As these results showed, one way to accomplish longer circulating lifetime of a prodrug is by increasing the  $M_w$  of the solubilizing agent to prevent rapid renal excretion of the hydrophilic form of the drug. It was also well established that large macromolecules which circulate for extended periods, show substantial tumor accumulation [64]. In fact, PEG molecules of  $10^4$  or greater  $M_w$  demonstrate a significantly higher ac-

cumulation in tumors than within normal tissue, irrespective of the tumor site [65]. The underlying physiological mechanism appears to be a combination of increased tumor vascular permeability and insufficient lymphatic drainage resulting in what is termed the “enhanced permeation and retention (EPR) effect”, which is thought to be an universal solid tumor phenomenon for macromolecular drugs. The EPR effect has been the subject of intensive studies for SMANCS [6] and HPMA Dox copolymer [66]. It was also shown that PEG-CPT prodrug (7, see Section 3.1.3) produced greater tumor accumulation (30 fold) in mice bearing subcutaneous tumors compared to unmodified CPT (Fig. 4) [67]. Not only is there an increase in the total dose of CPT delivered to the tumor, but the conjugate also provides higher tumor to normal tissue ratios of CPT over a longer period of time as compared to the administration of free CPT dispersed in an intralipid formulation. Whether the enhanced CPT uptake was in the form of the intact conjugate or released drug is unknown at this time. Thus, any long circulating prodrug that can be targeted to a tumor, to any degree, should show a higher degree of efficacy, and perhaps a greater therapeutic index. Therefore, PEG prodrugs may have distinct advantages over simple native drugs and prodrugs.

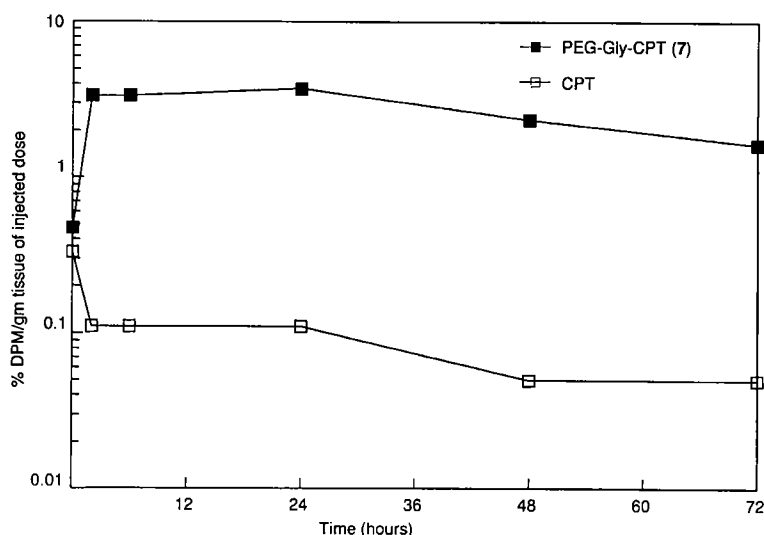


Fig. 4. Accumulation of labeled CPT in HT-29 tumors of nude mice (4 mice/group per time point) following a single i.v. injection of either PEG-gly- $^{[3]H}$ CPT or  $^{[3]H}$ CPT.

To validate the effect of  $M_w$ , ester prodrugs of paclitaxel and camptothecin (CPT) with similar  $t_{1/2}$  and  $IC_{50}$  but with variable  $M_w$  PEG (Fig. 3, 5 and 6) were also prepared. This allowed a clear distinction to be made as to whether or not relationship (1) was accurate using as the criterion the acute murine toxicity. CPT derivatives conjugated with PEG (6) 8000, 20,000 or 40,000 diacid (3) [68] were administered as a single i.v. injection to mice. All mice received the same amount of active CPT (25 mg/kg), but demonstrated considerable differences in levels of toxicity [38]. Lethality was approximately 50%, 10% and 0% for the PEG-CPT 40,000, 20,000 and 8000 constructs, respectively. As was noted, the hydrolysis rates within the PEG-CPT drug series were constant, thus the divergent levels of toxicity were probably due to differences in distribution, metabolism, and excretion. Urinary excretion studies support this theory. When equimolar amounts of PEG 40,000, 20,000 and 8000 diacids were administered i.v. in rats, an inverse correlation between  $M_w$  and excretion rate was observed (Fig. 5). All detectable PEG 8000 diacid was excreted within the first 6 h after dosing. In contrast, it took 24 h to recover all the PEG 20,000 diacid and over 96 h to excrete the PEG 40,000 diacid. Thus, the conjugate's  $M_w$  can have a profound impact on its systemic circulation. Early PEG-organic drug conjugates of  $M_w$  5000 or less [13] unfortunately were never tested in vivo and

it was not recognized at the time that  $M_w$  is an important feature of drug design; especially where there is only one site available for PEGylation. Ostensibly, employing polymer of  $M_w$  5000 to conjugate drugs gave rapidly excreted species that would have little or no effect in vivo. Application of PEG to most anticancer prodrugs mandates the use of polymer with a  $M_w$  of 30,000 or greater in order to prevent rapid elimination of the PEGylated species, and allow for passive tumor accumulation [6,64,67,69,70]. This bipartite prodrug approach has been employed by Enzon research groups extensively for the delivery of such cytotoxins as camptothecin, paclitaxel, and podophyllotoxin.

The first application of  $HM_w$  PEG to prodrugs was the synthesis of a PEG 40,000 ester of paclitaxel (5) using PEG acid, 3. In the earlier section, it was pointed out that a discrepancy existed between in vitro and in vivo efficacy for  $LM_w$  PEG paclitaxel (4). After it was established that acute toxicity resulted from high doses of 5 [61], the efficacy of the  $HM_w$  prodrug was re-examined. In a P388/O mouse leukemic model, compound 5 was found to be essentially equivalent to a TAXOL<sup>®</sup> formulation (Table 1). Of greater interest to the performance characteristics of the PEG-paclitaxel esters is the use of spacer molecules between the drug and the PEG ballast (Section 3.1.3).

Camptothecin (CPT) is an alkaloid isolated from

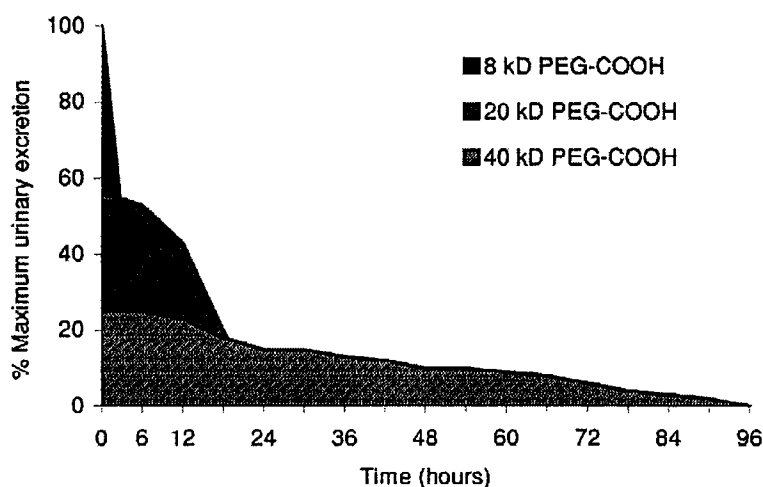
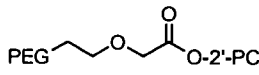
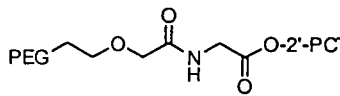


Fig. 5. Urinary elimination of PEG-COOH in rats following intravenous administration.

Table 1  
In vitro<sup>a</sup> and in vivo results of PEG-paclitaxel derivatives

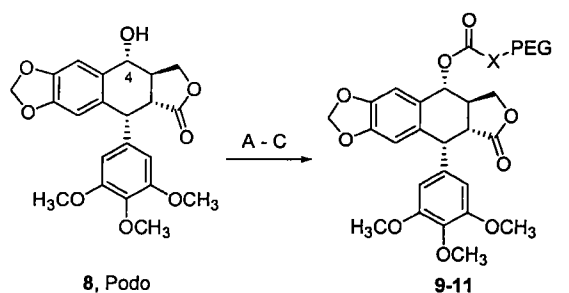
Compound	#	IC <sub>50</sub> (nM) P388/O	t <sub>1/2</sub> (h) <sup>b</sup>		P388 in vivo <sup>c</sup>				
			PBS pH 7.4	Rat plasma	Total dose (mg/kg)	Mean time to death (days) <sup>d</sup> [cures/group]	% ILS <sup>e</sup>	P values vs. control	P values vs. paclitaxel
Control	–	–	–	–	–	13.2±1.2 [0/10]	–	–	–
Paclitaxel	6	–	–	–	75	17.5±1.7 [0/10]	33	P=0.0151	–
					100	13.7±1.3 [0/10]	4	P=0.7714	–
	5	10	5.5	0.4	75	19.0±1.1 [0/10]	44	P=0.0013	P=0.3850 <sup>f</sup>
	22	14	7.0	0.4	75	21.8±1.0 [0/10]	65	P<0.0001	P=0.0151 <sup>f</sup>
					100	24.0±8.9 [1/10]	82	P<0.0001	P<0.0001 <sup>g</sup>

<sup>a</sup>All experiments were done in duplicate; Standard deviation of measurements = ±10%. <sup>b</sup>These results more appropriately, represent the half lives of disappearance of the transport form. <sup>c</sup>In vivo efficacy study of the water soluble paclitaxel derivatives using the P388/0 murine leukemia model. Paclitaxel or prodrug derivatives were given, in equivalent dose of paclitaxel, daily [intraperitoneal (i.p.)×5], 24 h following an injection of P388/0 cells into the abdominal cavity with survival monitored for 40 days. <sup>d</sup>Kaplan–Meier estimates with survivors censored. <sup>e</sup>Increased life span (%ILS) is (T/C – 1)×100. <sup>f</sup>Paclitaxel at 75 mg/kg. <sup>g</sup>Paclitaxel at 100 mg/kg.

the oriental tree, *Camptotheca acuminata*. CPT has the unique structural elements of a lactone ring and a 3° alcohol, both of which are requirements for its potent anticancer activity [71–73]. The biggest drawback to the use of this potent drug is that CPT is virtually insoluble in water and until recently the problem was approached by using a prodrug bearing an amino group as a salt in order to effect solubilization [74,75]. Using a PEG prodrug delivery strategy, it was reported that CPT can be solubilized as a non-ionic  $\alpha$ -alkoxyester conjugated to PEG carboxylic acid with a  $M_w$  of 40,000 [76]. CPT's solubility within the 20-CPT PEG 40,000 ester (PEG- $\alpha$ -CPT, **6**) prodrug form of minimally 2 mg/mL in water is dramatically greater than that of CPT (0.0025 mg/mL, water). **6** has been shown to hydrolyze in vivo and gradually release native CPT [77]. Fortuitously, it was found that modifying CPT at the 20 position as a PEG ester stabilizes the active lactone ring (essential for activity) under physiological conditions [76,78].

Podophyllotoxin (Podo, **8**), an aryltetralin lactone [79], is the principal active compound of the resin mixture known as podophyllin obtained from the dried roots of *Podophyllum peltatum* (also known as

American mandrake). Podo has a wide array of biological effects and is a spindle poison similar to colchicine and the vinca alkaloids and inhibits microtubule assembly by binding to tubulin during mitosis [80]. **8** is virtually insoluble in water, and early in vivo experiments were carried out by dissolution of **8** in either alcohol/water or propylene glycol followed by subcutaneous injection [81]. Clinicians and research scientists have long speculated that Podo's lackluster therapeutic index could be a result of its insolubility and unpredictable systemic behavior [82], which has generated interest in its modification. Early efforts [83] to reduce the toxicity of **8**, and maintain or enhance activity were directed along the lines of simple ester synthesis. While several of the esters showed significant activity, none was water soluble or demonstrated any increase in activity compared to Podo when tested at the same dosage level in a P388 lymphocytic leukemia mouse model. On the other hand, the solubilization utilizing PEG was accomplished [84] by reacting **8** with PEG derivatives to yield ester (**9**), carbonate (**10**), or carbamate (**11**) (Fig. 6). Although the PEG Podo analogs were highly water soluble, at best they were only slightly more efficacious than



Method	Product: X-PEG =
A: 3/ DIPC / DMAP	9:
B: PEG-CH <sub>2</sub> -O-CO-O-C <sub>6</sub> H <sub>4</sub> -NO <sub>2</sub> , DMAP PEG-PNP carbonate (12)	10:
C: PEG-CH <sub>2</sub> -N=C=O, DMAP PEG isocyanate (13)	11:

Fig. 6. PEG bipartate podophyllotoxin conjugates.

native drug. The reason for this lack of improvement may simply be due to Podo's innate mechanistic limitation to cause tumor cell cytotoxicity in vivo and higher toxicity resulting from the body's prolonged exposure to slowly released Podo within the circulation. From this report it would appear that  $HM_w$  PEG conjugated to antitumor agents does not always guarantee enhanced activity.

### 3.1.3. Enhancement of activity by employment of spacer groups

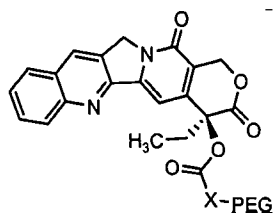
While the simple  $\alpha$ -alkoxy esters described for CPT and paclitaxel were efficacious, introduction of various spacer groups between the PEG solubilizing portion of the molecule and the drug alcohol was found to substantially change the plasma  $t_{1/2}$  and alter the therapeutic index [85]. This type of modification adds another dimension of consideration to synthetic design—especially in deciding at what point PEG conjugation should be carried out. Various spacer groups were employed and their application in CPT [86], paclitaxel [85], and Podo [84] were reported. The spacer groups were mainly bifunction-

al small moieties, especially heterobifunctional moieties including amino acids. One consequence of using linker groups was the introduction of rate accelerations due to neighboring group (anchimeric assistance, known for amides [87,88], carbamates [89], and amines [90,91]) participation. Although it was found that different spacers were preferred for different drugs, some predictive rules could be derived from the studies by comparing the rates of breakdown of the prodrugs in rat plasma with in vivo results.

As Table 2 shows, the rates of hydrolysis for various PEG-spacer-CPT prodrugs in PBS buffer (pH 7.4) showed considerable variation with  $t_{1/2}$  being as low as 0.2 h and as high as 102 h. In rat plasma the variation was less extreme, but nonetheless  $t_{1/2}$  clearly differentiated between spacer groups (e.g. 14 vs. 15) [86]. Thus, anchimeric assistance emerged as a primary mechanism in the hydrolytic process for those structures where an NH or NH(C=O) functionality was present, and a 3-, 5-, or 6-membered cyclic transition state could be formed with the terminal ester. Interestingly, it was reported that of the various spacer combinations tried, glycine yielded a water soluble prodrug with equipotent activity to the simple ester, a longer circulatory  $t_{1/2}$ , and less in vivo toxicity [67]. The judicious choice of using esters of simple amino acids as spacer groups coupled to PEG, seemed sufficient for adjusting the breakdown of the conjugate [86]. It appeared that by altering the amino acid spacer group within the conjugate, steric hindrance resulting from  $\alpha$ -substitution could lead to different rates of both enzymatic and non-enzymatic breakdown. In this way, the rate of dissociation can be taken to the extremes: exclusively rapid circulatory hydrolysis as opposed to only slow intracellular breakdown. Both extremes are faulty, since rapid circulatory hydrolysis can result in a "spiking" effect that can be profoundly toxic to normal cells, while most intracellular breakdown depends on unpredictable endocytotic transport and slow enzymatic degradation [92]. In summary, changes in kinetics (in vitro) also appear to have a significant effect on both the safety and efficacy of the drug in vivo [93].

It is also possible that specific amino acids may result in favorable hydrolysis of the ester bond between the amino acid and CPT via pH or esterase

Table 2

In vitro<sup>a</sup> and in vivo results of PEG-camptothecin derivatives

X-PEG	#	IC <sub>50</sub> (nM) P388/O	t <sub>1/2</sub> (h) <sup>b</sup>		P388 in vivo <sup>c</sup>		
			PBS pH 7.4	Rat plasma	Mean time to death (days) <sup>d</sup>	%ILS <sup>e</sup>	Survivors on day 40
Control		–	–	–	13.0	–	0/10
Camptothecin		7	–	–	38.0*	192	7/10
	6	15	27	2	38.0*	192	9/10
	14	16	5.5	0.8	17.4 <sup>f</sup>	34	4/10
	15	21	27	3	31.6* <sup>f</sup>	143	6/10
	16	18	28	5	23.4	80	0/10
	17	12	40	6	35.0*	169	8/10
	18	15	97	10	19.3* <sup>f</sup>	48	0/10
	19	24	12	3	30.6*	135	0/10
	20	42	102	>24	21.4* <sup>f</sup>	65	0/10

<sup>a</sup>All experiments were done in duplicate; Standard deviation of measurements =  $\pm 10\%$ . <sup>b</sup>These results more appropriately, represent the half lives of disappearance of the transport form. <sup>c</sup>In vivo efficacy study of the water soluble camptothecin derivatives using the P388/0 murine leukemia model. Camptothecin or prodrug derivatives were given in equivalent dose of camptothecin (total dose of 16 mg/kg) daily [intraperitoneal  $\times 5$ ], 24 h following an injection of P388/0 cells into the abdominal cavity with survival monitored for 40 days. <sup>d</sup>Kaplan–Meier estimates with survivors censored. <sup>e</sup>Increased life span (%ILS) is  $(T/C - 1) \times 100$ . \*Significant ( $P < 0.001$ ) compared to control (untreated). <sup>f</sup>Significant ( $P < 0.001$ ) compared to camptothecin.

mediated release. In contrast, others may initially encourage amide bond breakage between PEG and the amino acid by exo-peptidases or proteinases in the tumor resulting in an amino acid–CPT ester conjugate which would still have its bioavailability enhanced by lactone stabilization. The ester bond would subsequently be cleaved to release CPT. Interestingly, two recent papers have reported on the cellular uptake of amino acid ester prodrugs by a peptide transporter, regardless of the fact that no peptide bond is present in their structure [94,95]. Thus, entrance into the cell may theoretically be possible through either active or passive transport. Further delineation of potential tripartate systems would be a worthwhile undertaking. Cross species differences affecting rates of dissociation of the prodrug also need to be considered when examining the usefulness of any conjugate. Clinical results have been reported for PROTHECAN<sup>®</sup>, a conjugate of PEG to CPT via alanine [96], a hindered and relatively stable spacer in buffer but is hydrolyzed in human serum with a  $t_{1/2}$  of about 7 h (in vitro, 37 °C). Free CPT levels in plasma were measured in a Phase I study in patients with various types of solid tumors. Maximal levels of free CPT were observed about 24 h after infusion, reflecting the interplay between release of CPT from the PEG conjugate, clearance of the conjugate, and clearance of the released CPT. The maximal concentration of free camptothecin was proportional to the dose of PEG-CPT, reaching about 0.5  $\mu\text{g/mL}$  at the MTD of 120  $\text{mg/m}^2$  of CPT (equivalent to 7  $\text{g/m}^2$  of PEG-CPT conjugate). By contrast, patients receiving similar doses of CPT in the form of sodium camptothecin in earlier clinical trials had maximal plasma free CPT concentrations in the range of 30–60  $\mu\text{g/mL}$ .

The strikingly high maximal plasma concentration of CPT may have accounted for the poor tolerability of sodium camptothecin in the earlier clinical trials. Anti-cancer activity in the form of partial tumor responses and prolonged stable disease was observed in Phase I trials of PEG-CPT, prompting initiation of Phase II trials in several solid tumor indications.

PEG paclitaxel prodrug strategies were also extended to spaced prodrugs [85] which require the use of heterobifunctional spacer groups [97–99] in a similar manner as was applied to CPT (Fig. 7). **22** (solubility  $\sim 125 \text{ mg/mL}$ , or 5 mg paclitaxel equivalent/mL) demonstrated less toxicity and enhanced in vivo antitumor activity compared with the native drug and the simple ester **5**. Another method for delivery of paclitaxel that utilizes PEG has been developed by Dosio and coworkers [100]. They reported that human serum albumin (HSA) conjugated to paclitaxel via a succinic spacer acted as a prodrug, and wished to further increase the  $t_{1/2}$  of the conjugate as well as to reduce macrophage uptake. This was accomplished by further conjugating the HSA portion of the paclitaxel conjugate with mPEG through a thioimide bond. The total clearance was reduced to 1.41 mL/h for the  $M_w$  5000 mPEG conjugate from 2.9 mL/h for the unPEGylated prodrug. Organ uptake, especially by liver and spleen, was reduced as well.

Since formation of prodrugs using a glycine spacer in the PEG paclitaxel and PEG-CPT prodrug transport forms has been shown to enhance efficacy in several animal models, a delivery strategy that utilized amino acid spacer groups also seemed warranted for Podo (**8**) [84]. A series of Podo 4-amino acid esters conjugated to PEG were reported (Fig. 8). All of the conjugates exhibited aqueous

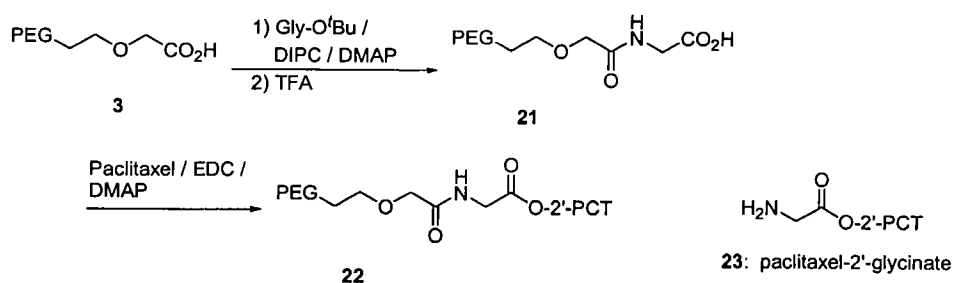


Fig. 7. PEG spaced esters.



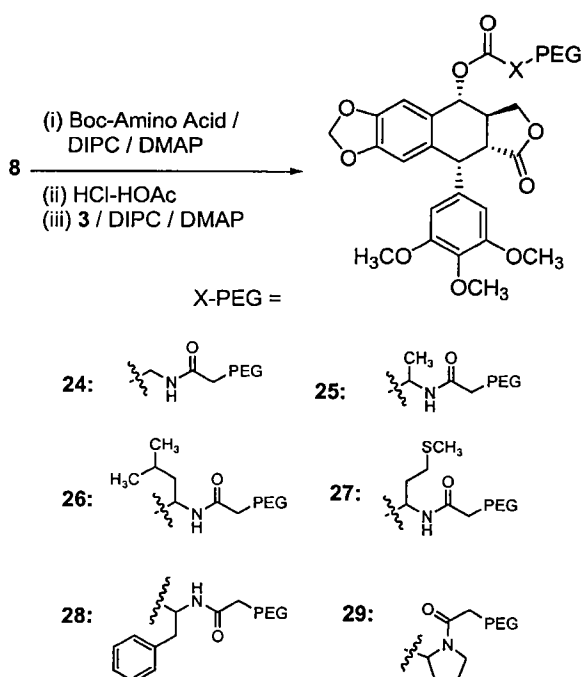


Fig. 8. PEG spacer prodrug conjugates.

solubilities similar to unfunctionalized PEG of  $M_w$  40,000, approximately 120–150 mg/mL. For the various  $\alpha$ -amino acid spacer groups employed, the  $IC_{50}$  and hydrolysis data appeared to parallel each other. However, in contrast to PEG-paclitaxel and PEG-CPT transport forms, which demonstrate a correlation between slower hydrolysis and improved therapeutic index against solid tumors, the slow sustained release of Podo appeared to reduce efficacy and increase toxicity in tumor bearing mice.

#### 3.1.4. Hydrazone–acid activated PEG–drug conjugates

A novel series of high  $M_w$  PEG conjugates that incorporated acid sensitive hydrazone linkages have been synthesized using PEG 20,000 and 70,000 [101]. Thus, Dox maleimide derivatives containing an acid-sensitive hydrazone linker, and a stable amide linker for comparison, were coupled to PEG using thiopropionic acid spacers as shown in Fig. 9. The polymer drug derivatives were designed to release Dox inside a tumor cell by acid-cleavage of the hydrazone bond, after endocytic cellular uptake.

The hydrazone conjugates demonstrated activity in vitro, albeit the activity was much less than Dox itself. In contrast, PEG Dox conjugates containing a stable amide bond at the amino sugar (3'-position) showed no in vitro activity at all. Fluorescence microscopy studies revealed that free Dox accumulates in the cell nucleus whereas the acid labile PEG-Dox derivatives are primarily localized in the cytoplasm.

#### 3.2. Tripartate PEG conjugate

While most amine drugs can be solubilized as acid salts, their rate of renal excretion is high. When converted to neutral small prodrug species, the ability to form salts is lost, and solubility may again become problematic. Not so in the case of PEG-drug conjugates, where PEG confers water solubility on insoluble small organic compounds without the need for forming salts. A general methodology for synthesizing PEG prodrugs of amino containing compounds has been developed [102,103] and constitutes the basis for solubilization of insoluble drugs while extending the plasma  $t_{1/2}$  of the prodrug. In the case of anticancer agents, apparent tumor accumulation also results. This novel PEG prodrug methodology can be accomplished in a rapid and facile manner.

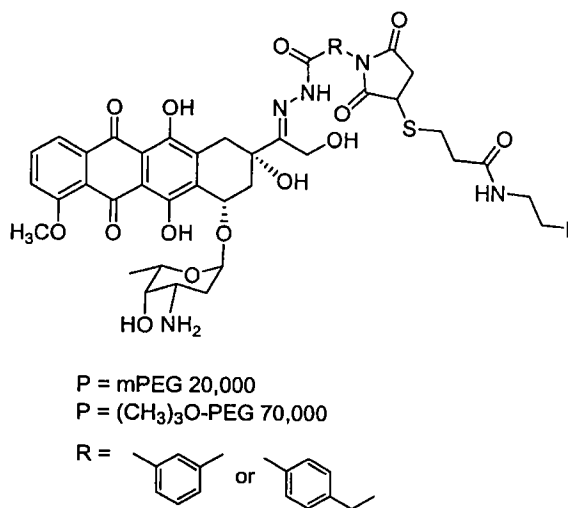


Fig. 9. PEG hydrazone drug conjugates.

### 3.2.1. Benzyl elimination (BE) system

Successfully designed PEG conjugated specifiers [104] or “triggers” [105] were synthesized as part of a double prodrug strategy that relied on enzymatic separation of PEG followed by the classical and rapid 1,4- or 1,6-BE reaction, releasing the amine (drug) initially bound (or latentiated) in the form of a carbamate (Fig. 10) [106]. This release technology has been developed extensively and is generally referred to as the double prodrug approach [58] since in essence a pro-prodrug has been made. In such systems, the hydrolytic sequence involves a first step which usually is an enzymatic cleavage, followed by a second, faster step, which is a molecular decomposition. Further refinement of the hydrolytic decomposition was accomplished by the introduction of steric hindrance through the use of ortho substituents on the benzyl component of the prodrug. This modification led to longer plasma  $t_{1/2}$  of the final tripartate form. The “ortho” effect also had the beneficial effect of directing nucleophilic attack almost exclusively to the activated benzyl 6-position of the heterobifunctional intermediates. This technology has extended the usefulness of the PEG prodrug strategy to amino containing anticancer compounds; it was also felt that the methodology would be applicable to other amino-drugs of diverse activity.

The efficacy of PEG-daunorubicin (DNR) conjugates prepared using the BE methodology was tested within a solid M109 tumor model and their relative activities varied according to route of administration and their rate of in vitro dissociation (Table 3). When the compounds were dosed i.p., the greatest activity was observed for native DNR followed by carbamate derivatives (34–36). However, when the PEG pro-

drugs were administered by the more clinically relevant i.v. route, those compounds with a rat plasma dissociation  $t_{1/2}$  of 2–4 h predominantly were effective in inhibiting solid tumor growth without causing toxicity and displayed a lower %T/C (i.e. greater anti-tumor effect) than an equivalent dose of DNR. The reason behind this phenomenon probably lies in the biodistribution of the PEG-drug conjugates, especially with respect to their rates of drug elimination versus tumor uptake.

Analogous conjugates to the DNR series were prepared for Dox [38] and, unexpectedly, gave a completely different profile (Table 4). For Dox, the aromatic amide derivative (40, analogous to 39 for DNR) provided the best results in a MX-1 model. This result clearly demonstrates that individual compounds must be subjected to a complete evaluation using different linkers in order to determine the most efficacious combination.

Drug delivery of insoluble agents using the BE elimination conjugation strategy with PEG was further explored with the antifungal agent amphotericin B (Fig. 11, AmB). This fungicide has such broad-spectrum activity that it remains the gold standard agent for many life-threatening fungal infections. However, AmB is virtually insoluble in water and can only be formulated into a suspension (FUNGIZONE®). A report of PEG conjugation to AmB has recently appeared [107]. Preliminary screening of in vitro antifungal activity of the permanently linked mPEG ( $M_w$  5000) urethane AmB derivative suggested that it has a similar spectrum of activity as the native drug formulated with sodium desoxycholate. In contrast prodrugs of AmB were prepared [108] using PEG ( $M_w$  40,000). Fortunately,

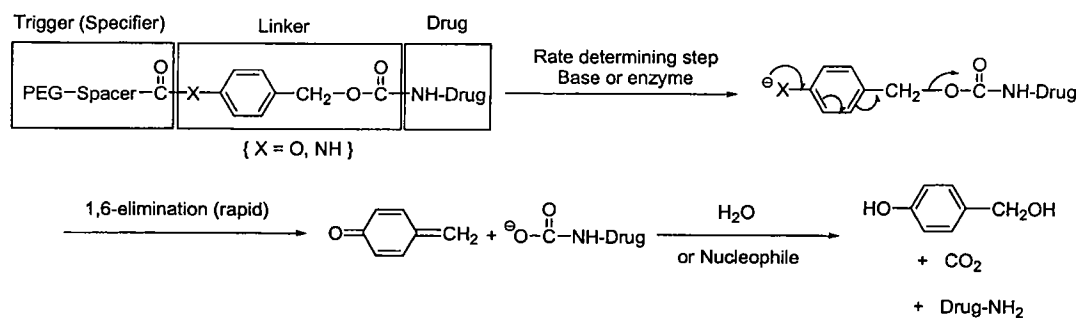
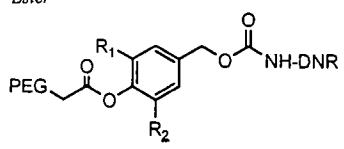
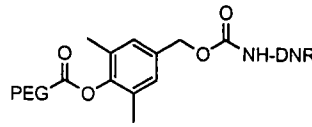
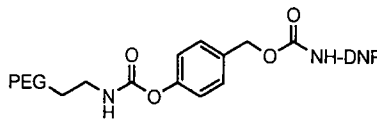
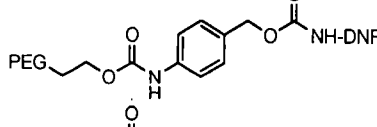
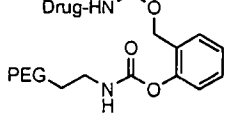
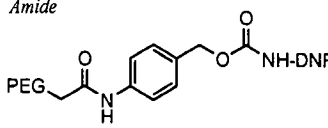
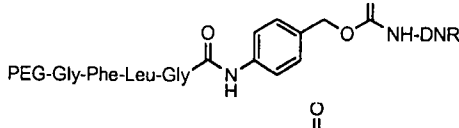
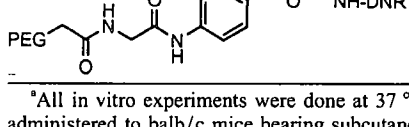


Fig. 10. PEG BE prodrug.

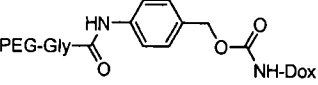
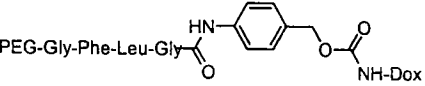
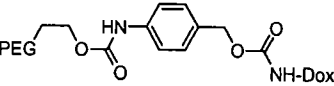
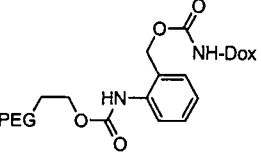
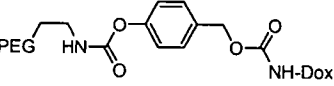
Table 3

In vitro and in vivo results of PEG-BE-Daunorubicin prodrugs

Compound	#	IC <sub>50</sub> (nM) P388/0	t <sub>1/2</sub> (h) <sup>a</sup>		M109 <sup>b</sup>	
			PBS (pH 7.4)	Rat plasma	i.p. %T/C	i.v. %T/C
Daunorubicin·HCl		3			44.8	117.0
<i>Ester</i>						
						
	30	8	>24	0.4	92.8	NA <sup>c</sup>
	31	27	>48	1.0	68.6	48.2
	32	55	>48	1.9	90.3	67.9
<i>Carbonate</i>						
	33	179	>48	2.9	90.3	74.4
<i>Carbamate</i>						
	34	15	>48	4	84.1	64.6
	35	415	>8	>24	75.3	129.0
	36	35	>48	3	91.3	68.7
<i>Amide</i>						
	37	457	>48	>24	122.7	NA <sup>c</sup>
	38	160	>24	13	87.6	82.6
	39	825	>24	>24	91.1	204.3

<sup>a</sup>All in vitro experiments were done at 37 °C in duplicate. Standard deviation of measurements = ±10%. <sup>b</sup>3 mg/kg/dose of active DNR administered to balb/c mice bearing subcutaneous Madison Lung Carcinoma on 1 & 4 (i.p.) or 3 & 6 (i.v.) days after inoculation. Percent treatment over control (% T/C) median tumor volumes were compared when control groups median tumor volume reached ~2000 mm<sup>3</sup>. <sup>c</sup>Result not available.

Table 4  
In vitro and in vivo results of PEG-BE-Doxorubicin prodrugs against MX-1<sup>a</sup>

Compound	#	$t_{1/2}$ (h, rat plasma)	Dose <sup>b</sup> (mg/kg) q7d×3, iv	T/C (%) <sup>c</sup> 1000 mm <sup>3</sup>
Doxorubicin		–	10	29.5
	40	6	10	69.6
	41	>24	10	68.2
	42	NA	10	114.1
	43	NA	10	71.4
	44	3	10	51.5

<sup>a</sup>Mean baseline (initial) tumor volume was approximately 100 mm<sup>3</sup> in this human mammary carcinoma (MX-1) xenograft model. <sup>b</sup>10 mg/kg/dose (Dox content) was given i.v. once a week for 3 weeks (Qd7×3). <sup>c</sup>Percent treatment over control (%T/C) median tumor volumes were compared when control group's median tumor volume reached 1000 mm<sup>3</sup>.

the amino group on the sugar ring of AmB, which is essential for its antifungal activity, provides an ideal site for PEG attachment as a promoiety. This investigation provided a series of di-substituted PEG-AmB derivatives which had in vitro PEG hydrolysis rates in plasma varying between 1 h and 3 h (Table 5). Importantly, all showed solubility greater than 30 mg/mL in aqueous media with good stability in PBS buffer. Efficacy studies in a *Candida albicans* infection model showed that both conjugates, **45** and **46**, when administered i.v. resulted in 100% survival at their MTD and 90% and 80% survival at 1/2 MTD, respectively. Negative and positive controls showed 10% of the vehicle control mice survived as compared to 70% of mice treated with 1 mg/kg AmB. As a major finding, this investigation of AmB demonstrated that its conjugation to PEG could

produce conjugates that were significantly (6×) less toxic than AmB-deoxycholate but maintained their in vivo antifungal effectiveness. Again, in this tripartate system, the alteration of PEG conjugate pharmacokinetics can be easily achieved by changing the PEG specifier and by adding a spacer and/or introducing steric hindrance. Thus greater drug efficacy could possibly be accomplished.

6-Mercaptopurine (6-MP, Fig. 11) is an orally administered highly insoluble purine analog that is effective against lymphatic leukemia. Oral absorption of 6-MP however is erratic with only 16 to 50% of the administered dose reaching the systemic circulation [109]. Water soluble PEG prodrugs of 6-MP were synthesized (Fig. 12) using several chemical schemes that enable the protection of the thiol group with spacers including a modified 1,6-BE

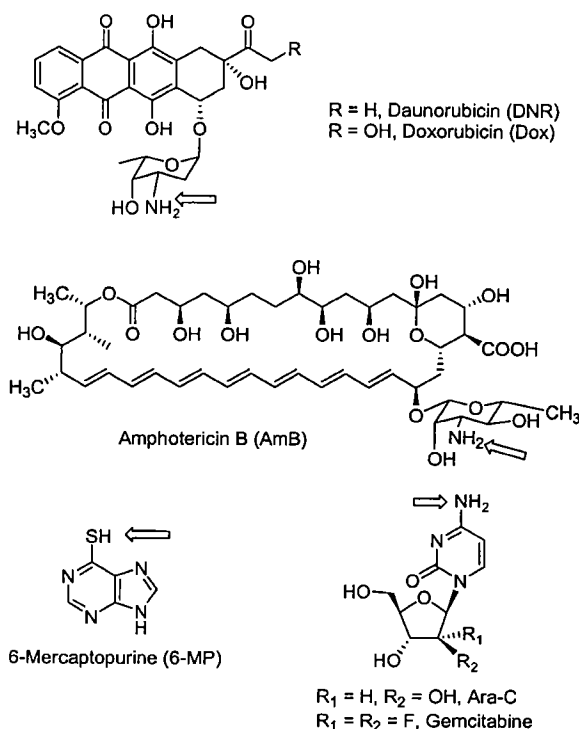


Fig. 11. Structure of drugs for tripartate prodrug and the prodrug site.

system [110]. PEG conjugation greatly enhanced the solubility of 6-MP, thus allowing straightforward formulation for i.v. administration. Efficacy testing

(Table 6) clearly demonstrated that some PEG 6-MP analogs had equivalent or superior anti-cancer activity compared to 6-MP in a solubilizing vehicle. Interestingly, increased 6-MP loading (tetramer vs. octamer) did not significantly affect the efficacy of the compound when compared on an equivalent 6-MP molar basis. The fact that some PEG 6-MP conjugates were stable in in vitro plasma dissociation assays, but demonstrated in vivo anti-cancer activity suggests possible hepatic cleavage of the linking group. As in most cases the exact mechanism by which this PEG-conjugate produced the enhanced anti-cancer activity in these models is not entirely clear.

### 3.2.2. Trimethyl Lock Lactonization (TML)

During the exploration of the limits of the PEG prodrug strategy, it was apparent that the use of lactonization reactions [111–113] could be incorporated into the strategy, and would provide a practical alternative to BE system. Researchers at Enzon have reported extended PEG technology which embraced the concept of the TML tripartate [114] system (Fig. 13). In order to utilize the TML system for polymer conjugated prodrugs, similar to BE system, it was necessary to first establish various methodologies which allowed the efficient synthesis of different acyl functionalities (triggers) such as esters, carbonates, and carbamates on the phenolic hydroxyl group. The acylating agents were by necessity bifunctional

Table 5  
In vitro and in vivo profile of PEG conjugated amphotericin B

Compound	#	Solubility in saline (mg/mL)	$t_{1/2}$ (h, rat plasma) <sup>b</sup>	Single dose <sup>c</sup> MTD in mice (mg/kg) <sup>d</sup>	In vivo survival at 1/2MTD
Amphotericin B (AmB)		<0.01	–	2	70
	45	31.2 [1.4] <sup>a</sup>	3.0	6	90
	46	56.3 [2.5]	1.5	12.5	80

<sup>a</sup>[<sup>a</sup>] Solubility of amphotericin B in conjugate (mg/ml). <sup>b</sup>PEG-amphotericin conjugate dissociation rate in vitro. <sup>c</sup>Based on Amphotericin B content. <sup>d</sup>Maximum tolerated dose based on body weight loss. The in vivo therapeutic efficacy of conjugates was studied with mice infected with *Candida albicans*.

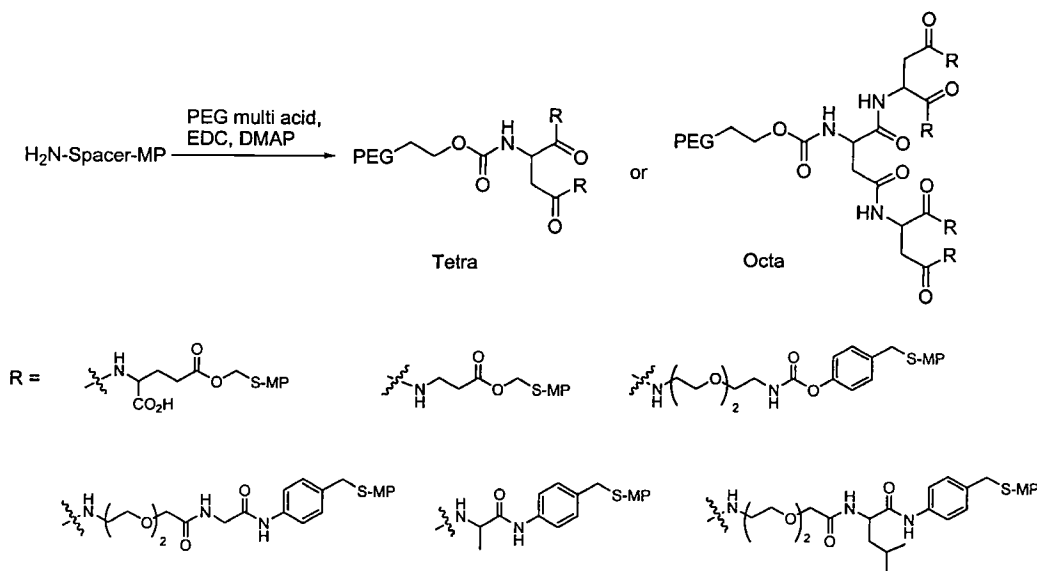


Fig. 12. Synthesis of tetrasubstituted PEG-spacer-S-drug conjugates.

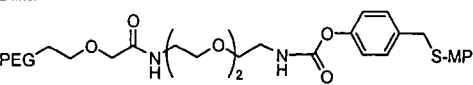
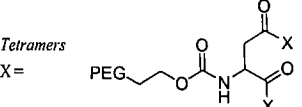
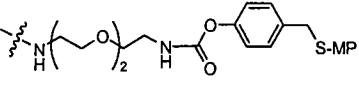
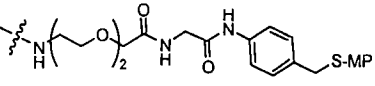
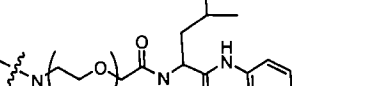
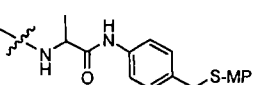
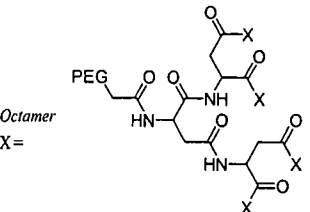
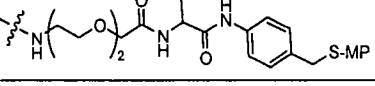
and offered a site for easy PEGylation. Thus, introduction of PEG into the TML system as part of the specifier or trigger resulted in a neutral and highly water soluble tripartate polymeric prodrug potentially capable of passive tumor targeting. The PEG prodrugs were designed to attain predictable rates of hydrolysis by changing the nature of the trigger/linker bond, by adding steric hindrance on the aromatic ring of the linker, and by the use of spacer groups. This approach resulted in a versatile methodology for easily altering the final design of the prodrug: it enabled a “mix and match” of spacers, triggers, and linkers that were utilized in a meaningful manner, and ultimately provided optimal plasma  $t_{1/2}$  for delivery of different types of drugs.

From the hydrolysis result of PEG-TML-DNR conjugates in rat plasma (Table 7), it is evident that adjusting  $t_{1/2}$  of TML linkers is not as simple as was the case for the BE system. A common thread that runs through these derivatives, as well as for **53** which is also quite effective, is the incorporation of an amino acid ester spacer. As has been mentioned earlier (Section 3.1.3) amino acid conjugated species may demonstrate enhanced tumor uptake.

A comparable study between TML and BE was also reported. **53** and **34** which appeared to have the

closest hydrolysis rate ( $t_{1/2}$  = 2–4 h) were evaluated along with a very slow releasing carbamate derivative (**35**) for chemotherapeutic activity against a more established and slower growing solid tumor model, SKOV3 ovarian cancer (~70 mm<sup>3</sup>) xenograft using nude mice (Table 8). Both compounds **34** and **53** were quite similar in their ability to significantly inhibit the growth of SKOV3 tumors. Surprisingly, the slow hydrolyzing carbamate (**35**) also had an effect on these solid tumors with post treatment mean volumes less than those of DNR treated animals. Therefore, while the slow release of DNR from **35** was perhaps overwhelmed by the rapid division and necrosis of cells within fast growing tumor, its accumulation and slow release within slow growing tumors may have caused a more therapeutic effect. In contrast, PEG conjugation did not appear to enhance the activity of DNR against tumor lines which were insensitive to DNR (MX-1; mammary and PC-3; prostate). That is not to say the attachment of PEG 40,000 with its ability to accumulate in tumors [65] will automatically permit drugs to have greater antitumor activity. Obviously, for PEG prodrug technology to be effective, the compound must have an adequate circulatory retention to allow ample tumor accumulation (of either the free drug or

Table 6  
Efficacy screen of PEG-6MP against sc M109 solid tumors in Balb/C mice

Compound	#	$t_{1/2}$ (h, rat plasma)	Treatment schedule <sup>a</sup> (mg/kg/dose <sup>b</sup> )	T/C (%) <sup>c</sup> at 1000 mm <sup>3</sup>
6-Mercaptopurine			d1 & 4 (30) d1 & 4 (100)	127.1 115.0
<i>Dimer</i>				
	47	2.0	d1 & 4 (30)	43.4
<i>Tetramers</i>				
				
	48	0.7	d1 & 4 (30)	27.9
	49	>24.0	d1 & 4 (30)	107.6
	50	15.2	d1 & 4 (30)	135.5
	51	>24.0	d1 & 4 (30)	33.9
<i>Octamer</i>				
				
	52	>24.0	d1 & 4 (30)	83.9

<sup>a</sup>Intravenous treatment of balb/c mice bearing non-established subcutaneous tumors. <sup>b</sup>Based on 6-MP content. <sup>c</sup>The median tumor volume of treatment and control groups were measured and compared when the control group's median tumor volume reached approximately 1000 mm<sup>3</sup>.

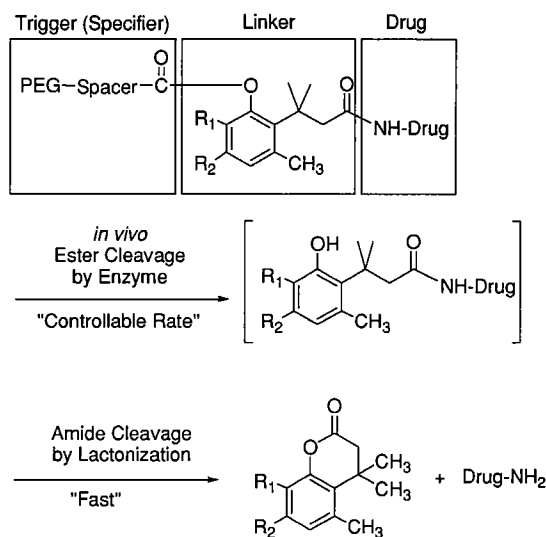


Fig. 13. PEG TML prodrug.

the conjugate) and if the entire conjugate is taken up it must contain a trigger that is cleaved within either the stromal environment or the neoplastic cells.

### 3.2.3. Tripartate delivery of bipartate prodrugs (tetrapartate system)

It is also possible to apply the tripartite system to an already existing amino bipartate prodrug. In essence this creates a tetrapartate prodrug. The concept can be illustrated using the known bipartate prodrug, Leu-Dox, which ultimately breaks down to native Dox, but is reported to have less cardiotoxicity. Attachment of the  $\alpha$ -amino group of Leu-Dox to the BE linker as a carbamate (Fig. 14) provides a triple prodrug system [38]. Since a 40,000  $M_w$  PEG is part of the prodrug one would expect an EPR effect to enhance the efficacy of Leu-Dox. A series of PEG BE prodrugs was prepared and the results summarized in Table 9. Several of the tetrapartate compounds demonstrated significant activity versus Leu-Dox itself.

### 3.3. PEG prodrugs of water-soluble small molecule, Ara-C

Ara-C (cytosine arabinose, 1-( $\beta$ -D-arabinofuranosyl) cytosine, Fig. 11) is a pyrimidine nucleoside analog employed for the treatment of

acute and chronic human leukemias such as ALL, AML, and CML. Its clinical utility is severely limited by the catabolic action of cytosine nucleoside deaminases, which are widely distributed in both normal and cancerous tissue, and which give rise to the inactive metabolite 1-( $\beta$ -D-arabinofuranosyl) uracil (ara-U). As a consequence, ara-C has a very short plasma  $t_{1/2}$ , which necessitates continuous infusion to provide maximum therapeutic efficacy [115] which causes some side effects. In order to overcome ara-C's shortcomings, many prodrug strategies have been explored with varied degrees of success [116]. Carboxylic and phosphate esters in the 3' and 5' positions have been examined, with some in human trials. Acylation of the  $N^4$ -amino group leads to amide [117] or carbamate [118,119] prodrug forms due to the easy enzymatic hydrolysis of the aromatic acyl functionality.

Recently, site-specific attachment of PEG on  $N^4$ -amino group was reported [120] employing acyl thiazolidine thiones. Since ara-C itself shows little or no activity against most solid tumors, passive tumor accumulation of a PEG ara-C prodrug conjugate was expected to provide the means of substantially increasing anticancer activity for this drug. Some of the disubstituted PEG linked ara-C prodrugs (Fig. 15) are shown in Table 10 with their rates of *in vitro* hydrolysis and results from *in vivo* anti-tumor test. These disubstituted prodrugs were highly soluble ( $\sim 300$  mg/mL in water) and were engineered to vary in their rate of *in vitro* hydrolysis (release of ara-C in plasma) from approximately 1 h to 3 days. No clear correlation could be observed between hydrolysis rates and *in vitro* cancer cell growth inhibition. In contrast, as a general trend, those prodrugs that hydrolyzed either too quickly ( $< 2$  h) or too slowly ( $> 40$  h) showed less anti-tumor activity in the xenograft model examined. Due to the relatively low loading capacity of these disubstituted conjugates ( $\sim 1\%$  ara-C w/w) only 20 mg/kg of active ara-C could safely be given per dose (viscosity). In contrast, the optimal dose of Ara-C is approximately 100 mg/kg/dose in this model. One approach to address this problem would be to obtain higher loading of ara-C on a single PEG strand. Advances toward this goal focused on the development of new dendritic (branched) PEG linkers.

To accomplish the objective of multi loading,



### In vitro<sup>a</sup> and in vivo results of PEG TML Daunorubicin prodrugs

\*All in vitro experiments were done at 37 °C in duplicate. Standard deviation of measurements =  $\pm 10\%$ . <sup>b</sup> mg/kg/dose of active DNR administered to balb/c mice bearing subcutaneous Madison Lung Carcinoma on 1 & 4 (i.p.) or 3 & 6 (i.v.) days after inoculation. Percent treatment over control (%T/C) median tumor volumes were compared when control groups median tumor volume reached  $\sim 2000 \text{ mm}^3$ .

\*All in vitro experiments were done at 37 °C in duplicate. Standard deviation of measurements =  $\pm 10\%$ . <sup>b</sup> mg/kg/dose of active DNR administered to balb/c mice bearing subcutaneous Madison Lung Carcinoma on 1 & 4 (i.p.) or 3 & 6 (i.v.) days after inoculation. Percent treatment over control (%T/C) median tumor volumes were compared when control groups median tumor volume reached  $\sim 2000 \text{ mm}^3$ .

Table 8

Efficacy of PEG-Daunorubicin analogs against established subcutaneous human ovarian tumors<sup>a</sup>(SKOV3) in nude mice

Treatment <sup>b</sup> ( <i>n</i> = 5/treatment)	#	Mean tumor volume by day 30	% Tumor growth <sup>c</sup> ( $\Delta$ from initial) by day 30	Mean tumor volume <sup>d</sup> (%T/C)	Median tumor volume <sup>e</sup> (%T/C)
Control (untreated)		1291	1588	–	–
Daunorubicin·HCl		1154	1226	92	54
PEG-BE-DNR	34	154*	86*	12	11
PEG-BE-DNR	35	272	400	21	41
PEG-TML-DNR	53	148*	79*	12	5

<sup>a</sup>Mean baseline (initial) tumor volume was approximately 70 mm<sup>3</sup>. <sup>b</sup>3 mg/kg/dose (DNR content) given i.v. on q4d  $\times$  3. <sup>c</sup>% mean tumor volume change from initial based on individual tumors at day 30. <sup>d</sup>A comparison of treatment and control group's mean tumor volume on day 30. <sup>e</sup>The median tumor volume of treatment and control groups were measured and compared when the control group's median tumor volume reached approximately 1000 mm<sup>3</sup>. \*Significantly ( $P < 0.05$ ) different from control (untreated).

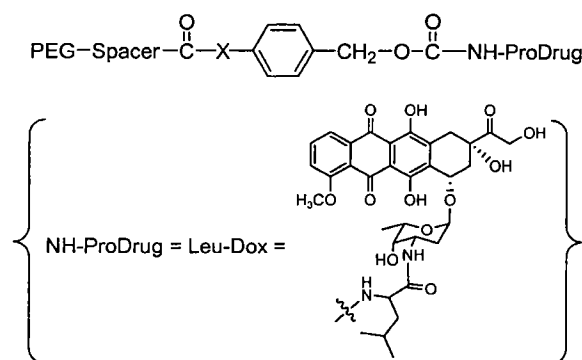


Fig. 14. PEG tetrapartate prodrug.

branching of the telechelic PEG polymer is required. Branching of the PEG termini could be accomplished in myriad ways; however, only components which could biodegrade to innocuous by-products would be desired. Using a strategy with a scaffolding of polyaspartic or polyglutamic acids linked to a bi-functional adamantane anchor, Ranganathan [121] linked pre-assembled Glu or Asp dendrons to achieve consecutive generations of two-directional peptidic dendrimers consisting of tetramers, octamers, and 16mers. This type of synthetic approach was well employed by the substitution of bifunctional PEG as the amphiphilic anchor [122]. However, conjugation of the dendritic PEG acid with Ara-C was achieved only after certain spatial requirements for the dendrimer-cytarabine conjugation were recognized and addressed. Apparently the steric bulk of several ara-C molecules in proximity to each other precluded the formation of the desired dendrimer. This problem was satisfactorily resolved by using an

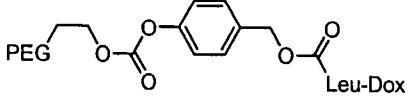
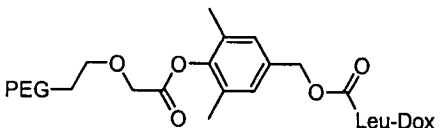
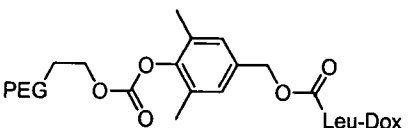
extended spacer, which further separated the branches of the dendron, allowing more complete conjugation to take place. It can be seen from Table 11 that both the tetramer and octamer are more soluble (and less viscous) than the double loaded derivative, a result which is probably due to the greater number of polyhydroxy sugars in the conjugates. As desired, the higher loading of drug provided substantial improvements in the LX-1 solid tumor model when compared to the disubstituted species. The octamer loaded PEG ara-C conjugates showed improved efficacy in two other in vivo cancer models, PANC-1 and P388/0. In summary, employing a multi-loaded branched PEG amide prodrug of ara-C substantially increased inhibition of tumor growth. This result demonstrated a potential threshold value exists for the amount of ara-C transported to the tumor site for optimal activity, which may be due in part to the presence of metabolic enzymes.

#### 3.4. Proprotein conjugates using releasable PEG (rPEG)

It is generally observed that protein functions, such as catalysis and receptor binding, are compromised to varying degrees following PEGylation, often resulting in diminished activity; this may be due to the presence of the PEG substituent on or near the protein's active or regulatory sites. Early attempts to improve this situation led to the use of different activated PEG linkers, all producing conjugates with hydrolysis-resistant permanent bonds with no one particular linker providing consistently superior results. Therefore, it seems likely that the most

Table 9

Efficacy summary of PEG-Leu-Doxorubicin against a human ovarian carcinoma (A2780) xenograft in nude mice<sup>a</sup>

Compound	#	$t_{1/2}$ (h, rat plasma) <sup>b</sup>	T/C (%) <sup>c</sup> at 1000 mm <sup>3</sup>
Leu-Dox		13	26.3
	59	2	13.2
	60	2.1	13.0
	61	3.3	14.3

<sup>a</sup>Intravenous treatment in nude mice bearing established tumors (~80 mm<sup>3</sup>). <sup>b</sup> $T_{1/2}$  was measured by the disappearance of the prodrugs, Leu-Dox or PEG conjugates, in the rat plasma at 37 °C. <sup>c</sup>30 mg/kg/dose (Dox content) was given intravenously once a week for 3 weeks (Qd7×3). The median tumor volume of treatment and control groups were measured and compared when the control group's median tumor volume reached approximately 1000 mm<sup>3</sup>.

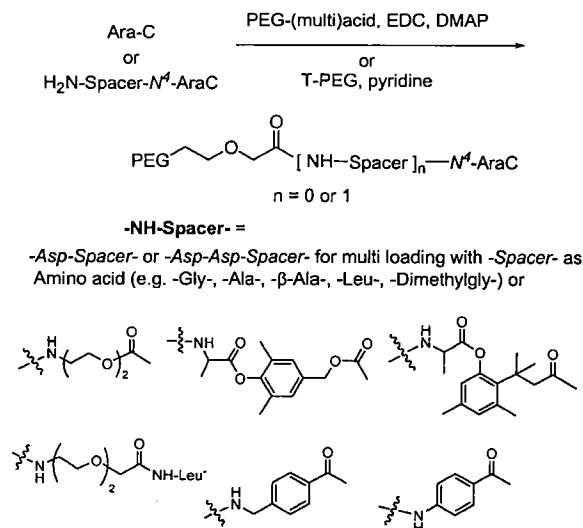


Fig. 15. PEG Ara-C conjugates.

effective linker for maintaining activity will vary for different proteins and will have to be determined empirically for each case [1,51]. PEG mass and the degree of protein modification have also been ex-

plored as a means of preserving activity; the use of fewer PEG strands of higher  $M_w$  has been reported to produce conjugates with less loss of activity [24,69]. A novel approach for maintaining maximum activity in a conjugate would be to design a functional PEG linker that can predictably break down by enzymatic or pH directed hydrolysis. Such a releasable PEG (rPEG) would provide rPEGylated protein-conjugates which are impermanent and could act as a depot or reservoir, continuously discharging native protein with full, albeit potentially short-acting, activity. BE derivatives based on mPEG 5000 (now termed rPEG) are ideal for application to protein and peptide modification using free  $\alpha$ - and  $\epsilon$ -amino functions, in the same fashion as small amino containing molecules to provide predictably unstable conjugates or prodrugs. Application of a non-PEGylated 1,6-BE prodrug strategy to N-Ras lipopeptides has been reported [123], but the conjugates appear to lack adequate water solubility and addition of co-solvents was found necessary in order to perform plasma experiments.

Demonstration of the PEG concept was accomplished using lysozyme as a model protein [124].

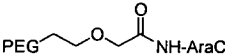
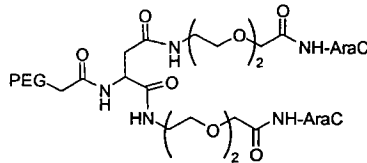
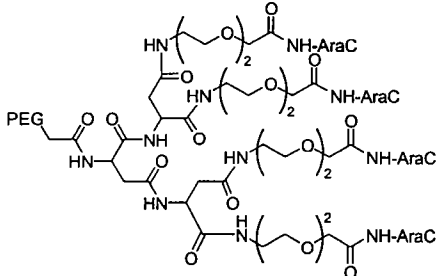
Table 10

In vitro and in vivo results of PEG Ara-C derivatives

Compound	#	$t_{1/2}$ (h, rat plasma) <sup>a</sup>	IC <sub>50</sub> (nM, P388/O) <sup>a</sup>	%TGI (LX-1) <sup>b</sup>
Ara-C	62	–	10	26.2
PEG-CH <sub>2</sub> CH <sub>2</sub> OCH <sub>2</sub> CO-NH-AraC	63	3	15	50.5
PEG-CH <sub>2</sub> CO-NH-CH <sub>2</sub> CO-NH-AraC	64	1.7	101	ND
PEG-CH <sub>2</sub> CO-NH-CH(CH <sub>3</sub> )CO-NH-AraC	65	1.5	88	ND
PEG-CH <sub>2</sub> CO-NH-CH <sub>2</sub> CH <sub>2</sub> CO-NH-AraC	66	16	196	36.9
PEG-CH <sub>2</sub> CO-NH-CH(CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub> )CO-NH-AraC	67	14	41	38.7
PEG-CH <sub>2</sub> CO-NH-C(CH <sub>3</sub> ) <sub>2</sub> CO-NH-AraC	68	92	297	10.5
PEG-CH <sub>2</sub> CO-NH-CH <sub>2</sub> -C <sub>6</sub> H <sub>4</sub> -CO-NH-AraC	69	65	122	12.3
PEG-CH <sub>2</sub> CO-NH-C <sub>6</sub> H <sub>4</sub> -CO-NH-AraC	70	75	1190	0
PEG-CH <sub>2</sub> CO-NH-CH(CH <sub>3</sub> )-O-C <sub>6</sub> H <sub>3</sub> (CH <sub>3</sub> ) <sub>2</sub> -C(CH <sub>3</sub> ) <sub>2</sub> -CO-NH-AraC	71	1.9	94	10.4
PEG-CH <sub>2</sub> CO-NH-(CH <sub>2</sub> CH <sub>2</sub> O) <sub>2</sub> -CO-NH-AraC	72	38	226	23.8

<sup>a</sup>All experiments were done at 37 °C in duplicate and  $t_{1/2}$  was measured by the disappearance of PEG derivatives. Standard deviation of measurements = ±10%. <sup>b</sup>Subcutaneous injections of LX-1 cells were allowed to reach an average tumor volume of 75 mm<sup>3</sup> prior to treatments (day 1). Ara-C (100 mg/kg/dose, optimal dose) and PEG-AraC derivatives (20 mg/kg/dose, volume limitation) were administered i.v. on day 1, 4, 7 and 10. Percent growth inhibition was calculated from the quotient of the median tumor volume of the treatment group divided by the median tumor volume of the control group  $\{(1 - T/C) \times 100\}$ . All PEG-AraC doses were based on their ara-C content. ND = not determined.

Table 11  
Summary of in vitro and in vivo results of PEG Ara-C

Compound	#	$t_{1/2}$ (h) <sup>a</sup> PBS, pH 7.4	$t_{1/2}$ (h) <sup>a</sup> human plasma	Solubility (mg/mL) <sup>b</sup>	Dose (mg/kg)	%TGI <sup>c</sup> (LX-1) solid tumor
Ara-C	62				100	26.2
	63	32	2.9	~300	20	50.5
	73	32	4.4	~400	40	66.3
	74	30	4.1	>500	60	78.2

<sup>a</sup>All experiments were done at 37 °C in duplicate and  $t_{1/2}$  was measured by the disappearance of PEG derivatives. Standard deviation of measurements =  $\pm 10\%$ . <sup>b</sup>Solubility in acidic formulated buffer. <sup>c</sup>Percent tumor growth inhibition (%TGI) was calculated from the quotient of the median tumor volume of the treatment group divided by the median tumor volume of the control group  $[(1 - T/C) \times 100]$  when the latter reached 1000 mm<sup>3</sup>.

Lysozyme, whose structure and function are well understood, also has the advantage that activity is totally lost upon permanent PEGylation. The presence of even a single PEG on its surface results in complete loss of enzymatic activity. This provides an opportunity to demonstrate unambiguously that PEG can be completely removed by regenerating the fully functional enzyme. The releasable PEG linker **75** (Fig. 16), an *N*-hydroxysuccinimidyl activated carbonate, was reacted with amines specifically at the alkyl terminus. The strategic placement of ortho methyl groups on the aromatic ring served to slow the rate of enzymatic cleavage of the PEG ballast and thus allowed a controlled release of native protein. The rate of release was compatible with the circulating  $t_{1/2}$  of permanently PEGylated proteins, which typically ranges from a few hours to several days [51,125]. In order for an rPEG protein conju-

gate to demonstrate effective drug delivery, activity must be regenerated before the protein is eliminated from the body by renal and hepatic clearance pathways. rPEG-lysozyme conjugates were relatively stable in pH 7.4 buffer for over 24 h. However, regeneration of native protein from the rPEG conjugates occurred in a predictable manner during incubation in high pH buffer or rat plasma as demonstrated by enzymatic activity and structural characterization.

Green fluorescent protein (GFP, **82**) was used as a model protein to study the in vivo release kinetics of various rPEGs [126]. GFP from the jellyfish *Aequorea victoria* is a natural fluorescent marker whose fluorescence intensity is not affected by PEGylation. This protein is ideal for monitoring pharmacokinetics, since rPEG-GFP concentrations in plasma can simply be determined by measuring the

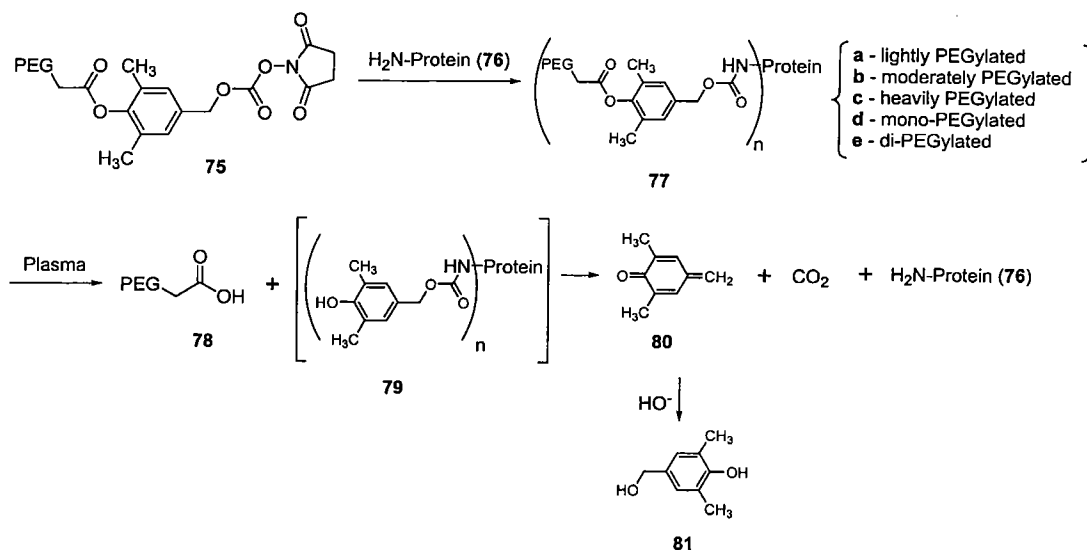
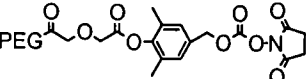
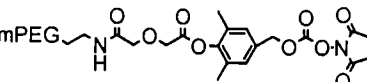
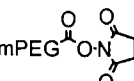


Fig. 16. rPEGylation of protein and release of protein from the rPEG-protein conjugates.

total plasma fluorescence intensity regardless of the number of PEG molecules on GFP or the presence of other plasma proteins. All purified rPEG-GFP conjugates used in the study are mPEG with an apparent  $M_w$  of 240,000. For pharmacokinetic studies, conscious rats were injected i.v. with 5 mg/kg of either GFP, permanent PEG-GFP (**85**) or two rPEG-GFPs

(83 & 84) and the fluorescence intensity of plasma samples was measured. Table 12 shows the conjugates' pharmacokinetics. Notice that the  $t_{1/2}$  of GFP in the rat was only 0.15 h, while the  $t_{1/2}$  of rPEG-GFP conjugates ranged from 1.8 to 10.6 h. As expected the PEG-GFP conjugate (85) derived from SC-PEG had the longest  $t_{1/2}$  (24.6 h).

Table 12  
Pharmacokinetics<sup>a</sup> of PEG-GFP conjugates (mean±S.E.)

PEG linkers	#	$t_{1/2}$ (h, rat plasma)	AUC
None (native GFP)	82	$0.15 \pm 0.03$	$0.15 \pm 0.02$
	83	$1.78 \pm 0.17$	$1.78 \pm 0.14$
	84	$10.61 \pm 1.30$	$14.82 \pm 1.62$
	85	$24.62 \pm 3.35$	$29.19 \pm 3.57$

<sup>a</sup>Pharmacokinetic parameters, plasma  $t_{1/2}$  and AUC (area under the curve), of PEG-GFP conjugate following a single i.v. bolus (5 mg/kg, GFP content) in rats.

### 3.5. PEG-peptide conjugates: PEG-DPDPE

One of the PEG-peptide conjugates that has been shown to act as a prodrug is PEG-DPDPE [127]. mPEG-SPA (succinimidyl propanoic ester,  $M_w$  2000) was conjugated to a met-enkephalin analog (DPDPE) and the pharmacodynamic and pharmacokinetic properties were studied in mice. PEG-DPDPE (i.v.) showed increased analgesia compared with the non-conjugated form despite a 172-fold lower binding affinity for the  $\delta$ -opioid receptor. Time course of distribution showed significant concentration differences in various organs between the conjugated and native forms. From the data presented it was concluded that PEG-DPDPE seems to act as a prodrug, enhancing peripheral pharmacokinetics, while undergoing hydrolysis in the brain and allowing non-conjugated DPDPE to act at the receptor.

### 3.6. Other applications of PEG prodrugs

Lele and Hoffman [128] designed a new mucohesive drug delivery formulation based on H-bonded complexes of poly(acrylic acid) (PAA) with the PEG of a prodrug, PEG-indomethacin conjugate. The PEGylated prodrug was synthesized with degradable PEG-anhydride-drug bonds, for eventual delivery of the free indomethacin from the formulation, by condensing PEG (5000) acid chloride with indomethacin in the presence of triethylamine. The resulting anhydride was surprisingly stable. The complexes are designed to dissociate as the formulation swells in contact with mucosal surfaces at pH 7.4, releasing PEG-indomethacin anhydride, which in turn then hydrolyzes to release free drug and PEG. It was determined that as the  $M_w$  of PAA increases, the dissociation rate of the complex decreases, which translates into a decreased rate of release of the native drug.

## 4. Hybrid rPEGylation of protein—a strategy

Typically, this technology involves rPEG-linkers that react with free amine groups present in the protein. In addition to serving as a releasable carrier, rPEG may also be used as a water soluble amino

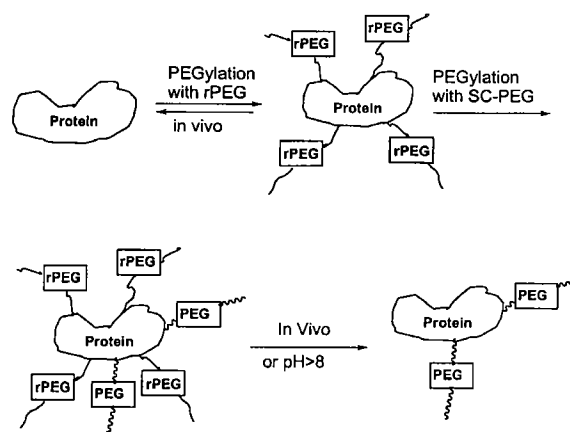


Fig. 17. Hybrid rPEGylation of protein.

protecting group on or near the active site of the protein. This can be followed by the attachment of permanently bound PEG at other sites where PEGylation is less critical to the biological activity of the protein to produce what can be termed a “PEG-hybrid” (Fig. 17). Once permanently bonded PEG is attached, the releasable PEG can then be removed during processing, leaving a non-immunogenic conjugate with fewer strands at active sites and therefore, greater activity. Alternatively, the hybrid conjugate can be used directly in vivo, further enhancing the duration of activity of the protein pharmaceutical [129,130]. This concept has been demonstrated using dimethylmaleic anhydride to block the active lysines of TNF- $\alpha$ , followed by PEGylation [131]. Pretreatment in this fashion gave conjugates with 20–40% higher specific activity in vitro than directly conjugated PEG-TNF- $\alpha$ .

## 5. PEG prodrug targeting

Novel PEG-immunoconjugates, recently reported by Yamasaki's group [132,133], have demonstrated antigen specific targeting using anticancer agents, DU-257 and adriamycin, with mAb, KM231 and NL-1, respectively. The enzymatically cleavable linker, PEG-L-ala-L-val, was coupled with DU-257, a potent anticancer duocarmycin derivative, through an amide bond. Coupling of the PEG-DU-257 conjugate to the KM231 mAb which is specifically reactive to

GD3 antigen, was then carried out. GD3 antigen is expressed on the surface of several malignant tumors such as SW1116, and in vitro testing using this cell line demonstrated significant growth inhibition at a concentration of 75  $\mu\text{g/mL}$ . Thus it appears that PEG-dipeptidyl prolinkers may be an effective means with which to prepare novel immunoconjugates.

## 6. Conclusion

For protein conjugation, early workers generally used  $LM_w$  mPEG (2000–5000) attached to multiple sites leading to long-lived protein conjugates. The early development of  $LM_w$  PEG proteins no doubt influenced small molecule drug delivery strategies, and probably accounts for  $HM_w$  PEG rarely being considered as pertinent for drug conjugates. However, during the past five years the field of PEG-drug conjugates has metamorphosed into an important delivery methodology, stimulated by the use of higher  $M_w$  PEGs. This recent resuscitation of small organic molecule delivery by  $HM_w$  PEG conjugates was founded on meaningful in vivo testing using established tumor models—not on misleading in vitro data or conjecture—and has resulted in the first reported example of a clinically relevant PEG-organic conjugate, PEG-camptothecin (PROTHECAN<sup>®</sup>), which is presently in Phase II clinical trials. The  $HM_w$  PEG-drug conjugates initially investigated were ester prodrugs, and refinement of ester chemistry led to the use of several different types of spacer groups between the drug to be modified and the PEG ballast. Furthermore, a very significant extension of the PEG prodrug strategy to amino and thiol groups has recently been realized using different approaches. These PEG prodrugs can be designed to attain predictable rates of hydrolysis by changing the nature of the trigger/linker bond, and by adding steric hindrance. These systems have additionally been modified to accommodate amide and carbamate triggers in order to allow passive tumor accumulation of the PEG prodrug to occur to a greater extent before breakdown. This approach offers the ability to exploit many combinations of triggers to achieve optimal pharmacokinetics for delivery of many types of drugs. However, studies

leading to a better understanding of their biodistribution and metabolism are warranted, but have yet to be reported.

The challenge of future work in the fascinating and useful world of  $HM_w$  PEG applications will not only be to continue the development of new anticancer prodrug clinical candidates, but additionally to extend the utility of this platform technology to the delivery of other classes of drug substances such as anti-fungals (amphotericin has been mentioned), antibiotics, immunosuppressives, and other bioactive agents. As of this review, encouraging reports of PEG applications to oligonucleotides, peptides and immunoconjugates for the purpose of drug delivery are appearing. While the PEGylation of proteins for purposes of enhanced drug delivery is expected to increase as new proteins become available, additional uses and novel strategies for employing PEG are just beginning. The list of future PEG applications, utilizing either high or low  $M_w$  polymers, tantalizes the imagination.

## Acknowledgements

We wish to thank Carol Aitken for her efforts in helping to prepare this manuscript. We also wish to acknowledge the outstanding contributions made by Drs. Chyi Lee, Hong Zhao, Kwok Shum, Stanford Lee, and Karen Yang. Special thanks to the Analytical and Pharm/Tox Groups of Enzon, Inc. for their outstanding performance in making much of this work possible.

## References

- [1] G. Hooftman, S. Herman, E. Schacht, Review: poly(ethylene glycol)s with reactive endgroups. II. Practical consideration for the preparation of protein-PEG conjugates, *J. Bioact. Compat. Polymers* 11 (1996) 135–159.
- [2] K.D. Vandegriff, M. McCarthy, R.J. Rohlf, R.M. Winslow, Colloid osmotic properties of modified hemoglobins: chemically cross-linked versus polyethylene glycol surface-conjugated, *Biophys. Chem.* 69 (1997) 23–30.
- [3] J.M. Harris (Ed.), *Poly(ethylene glycol) Chemistry, Biotechnical and Biomedical Applications*, Plenum Press, New York, 1992.
- [4] H.K. Roy, J.K. DiBaise, J. Black, W.J. Karolski, A. Ratashak, S. Ansari, *FEBS Lett.* 496 (2001) 143–146.



- [5] P.H. Toy, K.D. Janda, Soluble polymer-supported organic synthesis, *Acc. Chem. Res.* 33 (2000) 546–554.
- [6] H. Maeda, SMANCS and polymer-conjugated macromolecular drugs: Advantages in cancer chemotherapy, *Adv. Drug Delivery Rev.* 6 (1991) 181–202.
- [7] D. Putnam, J. Kopecek, Polymer conjugates with anticancer activity, *Adv. Polym. Sci.* 122 (1995) 55–123.
- [8] A. Bernstein, E. Hurwitz, R. Maron, R. Arnon, M. Sela, M. Wilchek, Higher antitumor efficacy of Daunomycin when linked to dextran: in vivo and in vitro studies, *J. Natl. Cancer Inst.* 60 (1978) 379–384.
- [9] S. Danhauser-Ried, E. Hausmann, H. Schick, R. Bender, H. Dietzfelbinger, J. Rastetter, A. Hanauske, Phase I clinical and pharmacokinetic trial of dextran conjugated Dox (AD-70, DOX-OXD), *Invest. New Drugs* 11 (1993) 187–195.
- [10] A. Jultani, C. Li, M. Ozen, M. Yadav, D. Yu, S. Wallace, S. Pathak, Paclitaxel and water-soluble poly (L-glutamic acid)-paclitaxel, induce direct chromosomal abnormalities and cell death in a murine metastatic melanoma cell line, *Anticancer Res.* 17 (1997) 4269–4274.
- [11] F. Zunino, F. Giuliani, G. Savi, T. Dasdia, R. Gambetta, Anti-tumor activity of Daunorubicin linked to poly-L-aspartic acid, *Int. J. Cancer* 30 (1982) 465–470.
- [12] Z.-R. Lu, P. Kopeček, Z. Wu, J. Kopeček, Functionalized semitelechelic poly[N-(2-hydroxypropyl) methacrylamide] for protein modification, *Bioconjugate Chem.* 9 (1998) 793–804.
- [13] M.L. Nucci, R. Shorr, A. Abuchowski, The therapeutic value of poly(ethylene glycol)-modified proteins, *Adv. Drug Delivery Rev.* 6 (1991) 133–151.
- [14] A. Abuchowski, T.J.R. McCoy, N.C. Palczuk, T. van Es, F.F. Davis, Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase, *J. Biol. Chem.* 252 (1977) 3582–3586.
- [15] S. Zalipsky, Chemistry of polyethylene glycol conjugates with biologically active molecules, *Adv. Drug Delivery Rev.* 16 (1995) 157–182.
- [16] R.B. Greenwald, A. Pendri, A. Martinez, C. Gilbert, R. Bradley, PEG thiazolidine-2-thione, a novel reagent for facile protein modification: conjugation of bovine hemoglobin, *Bioconjugate Chem.* 7 (1996) 638–641.
- [17] M.S. Hershfield, Adenosine deaminase deficiency: clinical expression, molecular basis, and therapy, *Semin. Hematol.* 35 (1998) 291–298.
- [18] S. David, A. Abuchowski, Y.K. Park, F.F. Davis, Alteration of the circulating life and antigenic properties of bovine adenosine deaminase in mice by attachment of polyethylene glycol, *Clin. Exp. Immunol.* 46 (1981) 649–652.
- [19] P.P.K. Ho, E.B. Milkin, J.L. Bobbitt, E.L. Grinnan, P.J. Burck, B.H. Frank, L.V.D. Boeck, R.W. Squires, Crystalline L-asparaginase from *Escherichia coli* B, *J. Biol. Chem.* 245 (1970) 3706–3715.
- [20] J. Kurtzberg, Asparaginase, in: J.F. Holland Jr, R.C. Bast Jr, D.L. Morton, E. Frei III, D.W. Kufe, R.R. Weichselbaum (Eds.), *Cancer Medicine* 4th Edition. Baltimore, MD, Lippincott Williams and Wilkins, VI, Section XVI, Chemotherapeutic Agents (1997) pp. 1027–1036.
- [21] Y. Wang, S. Youngster, J. Bausch, R. Zhang, C. McNemar, D.F. Wyss, Identification of the major positional isomer of PEGylated IFN alpha-2b, *Biochemistry* 39 (2000) 10634–10640.
- [22] M. Grace, S. Youngster, G. Gitlin, W. Sydor, L. Xie, L. Westreich, S. Jacobs, D. Brassard, J. Bausch, R. Bordens, Structural and biologic characterization of PEGylated recombinant IFN- $\alpha$ -2b, *J. IFN Cytokine Res.* 21 (2001) 1103–1115.
- [23] D.C. Wylie, M. Voloch, S. Lee, Y.H. Liu, S. Cannon-Carlson, C. Cutler, B. Pramanik, Carboxyalkylated histidine is a pH-dependent product of pegylation with SC-PEG, *Pharm. Res.* 18 (2001) 1354–1360.
- [24] R. Somack, M.G. Saifer, L.D. Williams, Preparation of long-acting superoxide dismutase using high molecular weight polyethylene glycol (41,000–72,000 daltons), *Free Radic. Res. Commun.* 12–13 (1991) 553–562.
- [25] S. Zeuzem, J.E. Heathcote, N. Martin, K. Nieforth, M. Modi, PegIFN alfa-2a (40 kDa) monotherapy: a novel agent for chronic hepatitis C therapy, *Expert Opin. Investig. Drugs* 10 (2001) 2201–2213.
- [26] A. Martinez, A. Pendri, R.B. Greenwald, Branched poly(ethylene glycol) linkers, *Macromol. Chem. Phys.* 198 (1997) 2489–2498.
- [27] C. Monfardini, O. Schiavon, P. Caliceti, M. Morpurgo, J.M. Harris, F.M. Veronese, A branched monomethoxypoly (ethylene glycol) for protein modification, *Bioconjugate Chem.* 6 (1995) 62–69.
- [28] G. Morstyn, M. Foote, T. Walker, G. Molineux, Filgrastim (r-metHuG-CSF) in the 21st century: SD/01, *Acta Haematol.* 105 (2001) 151–155.
- [29] F.A. Holmes, J.A. O'Shaughnessy, S. Vukelja, S.E. Jones, J. Shogan, M. Savin, J. Glaspy, M. Moore, L. Meza, I. Wiznitzer, T.A. Neumann, L.R. Hill, B.D. Liang, Blinded, randomized, multicenter study to evaluate single administration PEG filgrastim once per cycle versus daily filgrastim as an adjunct to chemotherapy in patients with high-risk stage II or stage III/IV breast cancer, *J. Clin. Oncol.* 20 (2002) 727–731.
- [30] J.C. Yang, S.L. Topalian, D.J. Schwartzentruber, D.R. Parkinson, F.M. Marincola, The use of polyethylene glycol-modified interleukin-2 (PEG-IL-2) in the treatment of patients with metastatic renal cell carcinoma and melanoma. A phase I study and randomized prospective study comparing IL-2 alone versus IL-2 combined with PEG-IL-2, *Cancer* 76 (1995) 687–694.
- [31] P.J. Trainer et al., Treatment of acromegaly with the growth hormone-receptor antagonist pegvisomant, *N. Engl. J. Med.* 342 (2000) 1171–1177.
- [32] R. Lustig, R. Byhardt, S. Diab, P. Lillis, P. Vigneri, J. Kwiatek, A.T. Viau, A clinical phase IB study of PEG-hemoglobin in patients receiving palliative fractionated radiation for solid tumors, *J. Clin. Oncology* 17 (1999) 1750.
- [33] D. Collen, P. Sinnaeve, E. Demarsin, H. Moreau, M.D. Maeyer, L. Jespers, Y. Laroche, F. Van de Werf, Polyethylene glycol-derivatized cysteine-substitution variants of recombinant staphylokinase for single-bolus treatment of acute myocardial infarction, *Cancer* 102 (2000) 1766–1772.

- [34] C. Delgado, G.E. Francis, D. Fisher, The uses and properties of PEG-linked proteins, *Critical Rev. Ther. Drug Carrier Syst.* 9 (1992) 249–304.
- [35] N.V. Katre, The conjugation of proteins with polyethylene glycol and other polymers, *Adv. Drug Delivery Rev.* 10 (1993) 91–114.
- [36] S. Zalipsky, C. Gilon, A. Zilkha, Attachment of drugs to polyethylene glycols, *Eur. Polym. J.* 19 (1983) 1177–1183.
- [37] R.B. Greenwald, A. Pendri, D. Bolikal, Highly water soluble taxol derivatives. 7-Polyethylene glycol carbamates and carbonates, *J. Org. Chem.* 60 (1995) 331–336.
- [38] Enzon, Inc. Unpublished results.
- [39] H.U. Esslinger, S. Haas, R. Maurer, A. Lassmann, K. Dübbers, H. Müller-Peltzer, Pharmacodynamic and safety results of PEG-Hirudin in healthy volunteers, *Thromb. Haemost.* 77 (1997) 911–919.
- [40] S.D. Yoo, H. Jun, B.S. Shin, H.S. Lee, M.O. Park, P.D. Deluca, K.C. Lee, Pharmacokinetic disposition of polyethylene glycol-modified salmon calcitonins in rats, *Chem. Pharm. Bull.* 48 (2000) 1921–1924.
- [41] M. Maeda, Y. Izuno, K. Kawasaki, Y. Kaneda, Y. Mu, Y. Tsutsumi, K.W. Lem, T. Mayumi, Amino acids and peptides, XXXII: A bifunctional poly (ethylene glycol) hybrid of fibronectin-related peptides, *Biochem. Biophys. Res. Comm.* 241 (1997) 595–598.
- [42] T.V. Chirila, P.E. Rakoczy, K.L. Garrett, X. Lou, I.J. Constable, The use of synthetic polymers for delivery of therapeutic antisense oligodeoxynucleotides, *Biomaterials* 23 (2002) 321–342.
- [43] A. Jaschke, J.P. Furste, D. Cech, V.A. Erdmann, Automated incorporation of polyethylene glycol into synthetic oligonucleotides, *Tetrahedron Lett.* 34 (1993) 301–304.
- [44] B. Seelig, A. Jaschke, Site-specific modification of enzymatically synthesized RNA: Transcription initiation and Diels–Alder reaction, *Tetrahedron Lett.* 38 (1997) 7729–7732.
- [45] T. Kawaguchi, H. Asakawa, Y. Tashiro, K. Juni, T. Sueishi, Stability, specific binding activity, and plasma concentration in mice of an oligodeoxynucleotide modified at 5'-terminal with poly(ethylene glycol), *Biol. Pharm. Bull.* 18 (1995) 474–476.
- [46] G.M. Bonora, E. Ivanova, V. Zarytova, B. Burcovich, F.M. Veronese, Synthesis and characterization of high-molecular mass polyethylene glycol-conjugated oligonucleotides, *Bioconjugate Chem.* 8 (1997) 793–797.
- [47] A. Jaschke, J.P. Furste, E. Nordhoff, F. Hillenkamp, D. Cech, V.A. Erdmann, synthesis and properties of oligodeoxyribonucleotide–polyethylene glycol conjugates, *Nucleic Acids Res.* 22 (1994) 4810–4817.
- [48] B. Burcovich, F.M. Veronese, V. Zarytova, G.M. Bonora, Branched polyethylene glycol (bPEG) conjugated antisense oligonucleotides, *Nucleos. Nucleot.* 17 (1998) 1567–1570.
- [49] P.E. Vorobjev, V.F. Zarytova, G.M. Bonora, Oligonucleotide conjugated to linear and branched high molecular weight polyethylene glycol as substrates for RNase H, *Nucleos. Nucleot.* 18 (1999) 2745–2750.
- [50] V. Rapozzi, S. Cogoi, P. Spessotto, A. Risso, G.M. Bonora, F. Quadrioglio, L.E. Xodo, Antigenic effect in K562 cells of a PEG-conjugated triplex-forming oligonucleotide targeted to the bcr/abl oncogene, *Biochemistry* 41 (2002) 502–510.
- [51] L.S. Lee, C. Conover, C. Shi, M. Whitlow, D. Filpula, Prolonged circulating lives of single-chain Fv proteins conjugated with polyethylene glycol: A comparison of conjugation chemistries and compounds, *Bioconjugate Chem.* 10 (1999) 973–981.
- [52] E. Hurwitz, L.N. Klapper, M. Wilchek, Y. Yarden, M. Sela, Inhibition of tumor growth by poly(ethylene glycol) derivatives of anti-ErbB2 antibodies, *Cancer Immunol. Immunother.* 49 (2000) 226–234.
- [53] I.L. Koumenis, Z. Shahrokh, S. Leong, V. Hsei, L. Deforge, G. Zapata, Modulating pharmacokinetics of an anti-interleukin-8 F(ab')<sub>2</sub> by amine-specific PEGylation with preserved bioactivity, *Int. J. Pharmaceut.* 198 (2000) 83–95.
- [54] T.-L. Cheng, B.-M. Chen, J.-W. Chern, M.-F. Wu, S.R. Roffler, Efficient clearance of poly(ethylene glycol)-modified immunoenzyme with anti-PEG monoclonal antibody for prodrug cancer therapy, *Bioconjugate Chem.* 11 (2000) 258–266.
- [55] V.J. Stella, W.N.A. Charman, V.H. Naringrekar, Prodrugs, do they have advantages in clinical practice?, *Drugs* 29 (1985) 455–473.
- [56] H. Bundgaard, Novel chemical approaches in prodrug design, *Drugs of the Future* 16 (1991) 443–458.
- [57] A.K. Sinhababu, D.R. Thakker, Prodrugs of anticancer agents, *Adv. Drug Delivery Rev.* 19 (1996) 241–273.
- [58] H. Bundgaard, The double prodrug concept and its applications, *Adv. Drug Delivery Rev.* 3 (1989) 39–65.
- [59] D.M. Vyas, Paclitaxel (Taxol®) formulation and prodrugs, in: V. Farina (Ed.), *The Chemistry and Pharmacology of Taxol and its Derivatives*, Elsevier Science B.V, 1995.
- [60] R.B. Greenwald, A. Pendri, D. Bolikal, C.W. Gilbert, Highly water soluble taxol derivatives: 2-polyethyleneglycol esters as potential prodrugs, *Bioorg. Med. Chem. Lett.* 4 (1994) 2465–2470.
- [61] R.B. Greenwald, C.W. Gilbert, A. Pendri, C.D. Conover, J. Xia, A. Martinez, Drug delivery systems: Water soluble taxol 2-poly (ethylene glycol) ester prodrugs-design and in vivo effectiveness, *J. Med. Chem.* 39 (1996) 424–431.
- [62] L.W. Seymour, R. Duncan, J. Strohalm, J. Kopecek, Effect of molecular weight ( $M_w$ ) of N-(2-hydroxypropyl) methacrylamide copolymers on body distribution and rate of excretion after subcutaneous, intraperitoneal, and i.v. administration to rats, *J. Biomed. Mat. Res.* 21 (1987) 1341–1358.
- [63] T. Yamaoka, Y. Tabata, Y. Ikada, Distribution and tissue uptake of polyethylene glycol with different molecular weights after i.v. administration to mice, *J. Pharm. Sci.* 83 (1994) 601–606.
- [64] Y. Noguchi, J. Wun, R. Duncan, R. Strohalm, K. Ulbrich, T. Akaike, H. Maeda, Early phase tumor accumulation of macromolecules: A great difference in clearance rate between tumor and normal tissues, *Jap. J. Cancer Res.* 89 (1998) 307–614.
- [65] Y. Murakami, Y. Tabata, Y. Ikada, Tumor accumulation of poly (ethylene glycol) with different molecular weights after i.v. injection, *Drug Del.* 4 (1997) 23–32.

- [66] J. Kopecek, R. Duncan, Targetable polymeric prodrugs, *J. Controlled Rel.* 6 (1987) 315–327.
- [67] C.D. Conover, R.B. Greenwald, A. Pendri, C.W. Gilbert, K.L. Shum, Camptothecin delivery systems: Enhanced efficacy and tumor accumulation of camptothecin following its conjugation to polyethylene glycol via a glycine linker, *Cancer Chemother. Pharmacol.* 42 (1998) 407–414.
- [68] A.J. Martinez, R.B. Greenwald, Method of preparing polyalkylene oxide carboxylic acids, U.S. Patent 5,605,976, 1997.
- [69] M.J. Knauf, D.P. Bell, P. Hirtzer, Z.P. Lou, J.D. Young, N.V. Katre, Relationship of effective molecular size to systemic clearance in rats of recombinant interleukin-2 chemically modified with water soluble polymers, *J. Biol. Chem.* 263 (1988) 15064–15070.
- [70] H. Maeda, L. Seymour, Y. Miyamoto, Conjugates of anti-cancer agents and polymers: Advantages of macromolecular therapeutics in vivo, *Bioconjugate Chem.* 3 (1992) 351–362.
- [71] M. Potmesil, Camptothecins: From bench research to hospital wards, *Cancer Res.* 54 (1994) 1431–1439.
- [72] A. Bedeschi, I. Candiani, C. Geroni, L. Capolongo, Water-soluble camptothecin derivatives, *Drugs Future* 22 (1997) 1259–1266.
- [73] M.E. Wall, M.C. Wani, Camptothecin and taxol: Discovery to clinic-Thirteenth Bruce F. Cain Memorial Award Lecture, *Cancer Res.* 55 (1995) 753–760.
- [74] B.R. Vishnuvajjala, A. Garzon-Aburbah, Water soluble prodrugs of camptothecin, U.S. Patent 4,943,579, 1990.
- [75] M.E. Wall, M.C. Wani, W. Nicholas, G. Manikumar, C. Tele, L. Moore, A. Truesdale, P. Leitner, J.M. Besterman, Plant antitumor agents. 30. Synthesis and structure activity of novel camptothecin analogs, *J. Med. Chem.* 36 (1993) 2689–2700.
- [76] R.B. Greenwald, A. Pendri, C. Conover, C. Gilbert, R. Yang, J. Xia, Drug delivery systems. 2. Camptothecin 20-*O*-poly(ethylene glycol) ester transport forms, *J. Med. Chem.* 39 (1996) 1938–1940.
- [77] C.D. Conover, A. Pendri, C. Lee, C.W. Gilbert, K.L. Shum, R.B. Greenwald, Camptothecin delivery systems: The antitumor activity of a camptothecin-20-*O*-polyethylene glycol ester transport form, *Anti Cancer Res.* 17 (1997) 3361–3368.
- [78] H. Zhao, C. Lee, P. Sai, Y.H. Choe, M. Boro, A. Pendri, S. Guan, R.B. Greenwald, 20-*O*-acylcampthothecin derivatives: evidence for lactone stabilization, *J. Org. Chem.* 65 (2000) 4601–4606.
- [79] R.S. Ward, The synthesis of lignans and neolignans, *Chem. Soc. Rev.* 11 (1982) 75–126.
- [80] D.L. Sackett, Podophyllotoxin, steganicin and combretastatin: Natural products that bind at the colchicine site of tubulin, *Pharmac. Ther.* 59 (1993) 163–228.
- [81] V. Seidlova-Masínová, J. Malinsky, F. Santavy, The biological effects of some podophyllin compounds and their dependence on chemical structure, *J. Natl. Cancer Inst.* 18 (1957) 359–371.
- [82] E.M. Greenspan, J. Colsky, E.B. Schoenbach, M.J. Shear, Response of patients with advanced neoplasms to the i.v. administration of  $\alpha$ -peltatin, *J. Natl. Cancer Inst.* 14 (1954) 1257–1275.
- [83] R.K. Levy, I.H. Hall, K.-H. Lee, Antitumor agents LXII: Synthesis and biological evaluation of podophyllotoxin esters and related derivatives, *J. Pharm. Sci.* 72 (1983) 1158–1161.
- [84] R.B. Greenwald, C.D. Conover, A. Pendri, Y.H. Choe, A. Martinez, D. Wu, S. Guan, Z. Yao, K.L. Shum, Drug delivery of anticancer agents: water soluble 4-poly(ethylene glycol) derivatives of the lignan, podophyllotoxin, *J. Control. Rel.* 61 (1999) 281–294.
- [85] A. Pendri, C.D. Conover, R.B. Greenwald, Antitumor activity of paclitaxel-2'-glycinate conjugated to poly(ethylene glycol): A water-soluble prodrug, *Anti-Cancer Drug Design* 13 (1998) 387–395.
- [86] R.B. Greenwald, A. Pendri, C.D. Conover, C. Lee, Y.H. Choe, C. Gilbert, A. Martinez, J. Xia, D. Wu, M. Hsue, Camptothecin-20-PEG ester transport forms: The effect of spacer groups on antitumor activity, *Bioorg. Med. Chem.* 6 (1998) 551–562.
- [87] S.A. Bernhard, A. Berger, J.H. Carter, E. Katchalski, M. Sela, Y. Shalitin, Co-operative effects of functional groups in peptides. I. Aspartyl-serine derivatives, *J. Am. Chem. Soc.* 84 (1962) 2421–2434.
- [88] B.M. Tadayoni, P.M. Friden, L.R. Walus, G.F. Musso, Synthesis, in vitro kinetics, and in vivo studies on protein conjugates of AZT: Evaluation as a transport system to increase brain delivery, *Bioconjugate Chem.* 4 (1993) 139–145.
- [89] U.S. Gogate, A.J. Repta, J. Alexander, *N*-(acyloxyalkoxycarbonyl) derivatives as potential prodrugs of amines. I. Kinetics and mechanism of degradation in aqueous solutions, *Int. J. Pharmaceut.* 40 (1987) 235–248.
- [90] T.H. Fife, B.R. DeMark, Intramolecular nucleophilic aminolysis of aliphatic esters. cyclization of methyl 2-amino-methylbenzoate to phthalimidine, *J. Am. Chem. Soc.* 98 (1976) 6978–6982.
- [91] W.S. Saari, J.E. Schwering, P.A. Lyle, S.J. Smith, E.L. Englehardt, Cyclization-activated prodrugs. Basic carbamates of 4-hydroxy anisole, *J. Med. Chem.* 33 (1990) 97–101.
- [92] H. Soye, E. Schacht, S. Vanderkerken, The crucial role of spacer groups in macromolecular prodrug design, *Adv. Drug Delivery Rev.* 21 (1996) 81–106.
- [93] C.D. Conover, R.B. Greenwald, A. Pendri, K.L. Shum, Camptothecin delivery systems: The utility of amino acid spacers for the conjugation of camptothecin with polyethylene glycol to create prodrugs, *Anti-Cancer Drug Design* 14 (1999) 499–506.
- [94] H. Han, D. Oh, G.L. Amidon, Cellular uptake mechanism of amino acid ester prodrugs in Caco-2/hPEPT1 cells over expressing a human peptide transporter, *Pharmaceut. Res.* 15 (1998) 1382–1386.
- [95] P.V. Balimane, I. Tamai, A. Guo, T. Nakanishi, H. Kitada, F.H. Leibach, A. Tsuji, P.J. Sinko, Direct evidence for peptide transporter (PepT1)-mediated uptake of a nonpeptide prodrug, valacyclovir, *Biochem Biophys. Res. Commun.* 250 (1998) 246–251.
- [96] L. Ochoa, A.W. Tolcher, J. Rizzo, G.H. Schwartz, A. Patnaik, L. Hammond, H. McCreery, L. Denis, M. Hidalgo, J. Kwiatek, J. McGuire, E.K. Rowinsky, A phase I study of

- PEG-camptothecin (PEG-CPT) in patients with advanced solid tumors: A novel formulation for an insoluble but active agent, *J. Clin. Oncol.* 19 (2000) 198a.
- [97] H.M. Deutsch, J.A. Glinski, M. Hernandez, R.D. Haugwitz, V. Narayan, M. Suffness, L.H. Zalkow, Synthesis of congeners and prodrugs. 3. Water-soluble prodrugs of taxol with potent antitumor activity, *J. Med. Chem.* 32 (1989) 788–792.
- [98] Z. Zhao, D.G.I. Kingston, A.R. Crosswell, Modified taxols, 6. Preparation of water-soluble prodrugs of taxol, *J. Nat. Prod.* 54 (1991) 1607–1611.
- [99] A.E. Matthew, M.R. Mejillano, J.P. Nath, R.H. Himes, V.J. Stella, Synthesis and evaluation of some water-soluble prodrugs and derivatives of taxol with antitumor activity, *J. Med. Chem.* 35 (1992) 145–151.
- [100] F. Dosio, S. Arpicco, P. Brusa, B. Stella, L. Cattel, Poly(ethylene glycol)-human serum albumin-paclitaxel conjugates: preparation, characterization and pharmacokinetics, *J. Controlled Rel.* 76 (2001) 107–117.
- [101] P.C.A. Rodrigues, U. Beyer, P. Schumacher, T. Roth, H.H. Fiebig, C. Unger, L. Messori, P. Orioli, E.H. Paper, R. Mulhaupt, F. Kratz, Acid-sensitive polyethylene glycol conjugates of doxorubicin: preparation, in vitro efficacy and intracellular distribution, *Bioorg. Med. Chem.* 7 (1999) 2517–2524.
- [102] R.B. Greenwald, A. Pendri, C.D. Conover, H. Zhao, Y.H. Choe, A. Martinez, K. Shum, S. Guan, Drug delivery systems employing 1,4- or 1,6-elimination: Poly(ethylene glycol) prodrugs of amine containing compounds, *J. Med. Chem.* 42 (1999) 3657–3667.
- [103] R.B. Greenwald, Y.H. Choe, C.D. Conover, H. Zhao, K. Shum, D. Wu, M. Royzen, Drug delivery systems based on trimethyl lock lactonization: Poly(ethylene glycol) prodrugs of amine containing compounds, *J. Med. Chem.* 43 (2000) 475–487.
- [104] P.L. Carl, P.K. Chakravarty, J.A. Katzenellenbogen, A novel connector linkage applicable in prodrug design, *J. Med. Chem.* 24 (1981) 479–480.
- [105] W.A. Denny, W.R. Wilson, The design of selectively-activated anti-cancer prodrugs for use in antibody-directed and gene-directed enzyme-prodrug therapies, *J. Pharm. Pharmacol.* 50 (1998) 387–394.
- [106] M. Wakselman, 1,4- and 1,6-eliminations from hydroxy and amino-substituted benzyl systems: Chemical and biochemical applications, *Nouveau J. de Chemie* 7 (1983) 439–447.
- [107] M. Sedlak, V. Buchta, L. Kubicova, P. Simunek, M. Holcapek, P. Kasparova, Synthesis and characterization of a new amphotericin B-methoxypoly(ethylene glycol) conjugate, *Bioorg. Med. Chem. Lett.* 11 (2001) 2833–2835.
- [108] H. Zhao, C.D. Conover, P. Reddy, A. Martinez, C.B. Longley, R.B. Greenwald, The Utility of Polyethylene Glycol Conjugation to Create Prodrugs of Amphotericin B, National Biotechnology Conference, June 24–26 (2002) San Diego, CA.
- [109] S. Zimm, J.M. Collins, R. Riccardi, D. O'Neill, P.K. Narang, B. Chabner, D.G. Poplack, Variable bioavailability of oral mercaptopurine. Is maintenance chemotherapy in a cute lymphoblastic leukemia being optimally delivered? *N. Engl. J. Med.* 308 (1983).
- [110] Y.H. Choe, S. Guan, R.B. Greenwald, PEG prodrugs of 6-mercaptopurine (6-MP)—II: Synthesis and in vitro studies, American Association for Cancer Research 93rd Annual Meeting (2002) April 6–10, San Francisco, CA.
- [111] D. Shan, M.G. Nicholaou, R.T. Borchardt, B. Wang, Prodrug strategies based on intramolecular cyclization reactions, *J. Pharm. Sci.* 86 (1997) 765–767.
- [112] B. Testa, J.M. Mayer, Design of intramolecularly activated prodrugs, *Drug Metab. Rev.* 30 (1998) 787–807.
- [113] W. Wang, J. Jiang, C.E. Ballard, B. Wang, Prodrug approaches to the improved delivery of peptide prodrugs, *Curr. Pharm. Des.* 5 (1999) 265–287.
- [114] R.B. Greenwald, Y.H. Choe, C.D. Conover, K. Shum, D. Wu, M. Royzen, Drug delivery systems based on trimethyl lock lactonization: Poly(ethylene glycol) prodrugs of amine containing compounds, *J. Med. Chem.* 43 (2000) 475–487.
- [115] A.F. Hadfield, A.C. Sartorelli, The pharmacology of prodrugs of 5-fluorouracil and 1- $\beta$ -D-arabinofuranosylcytosine, *Adv. Pharmacol. Chemother.* 20 (1984) 21–67.
- [116] P. Wipf, W. Li, Prodrugs of ara-C, *Drugs Future* 19 (1994) 49–54.
- [117] M. Aoshima, S. Tsukagoshi, Y. Sakurai, J. Oh-ishi, T. Ishida, H. Kobayashi,  $N^4$ -behenoyl-1- $\beta$ -D-arabinofuranosylcytosine as a potential new antitumor agent, *Cancer Res.* 37 (1977) 2481–2486.
- [118] T.A. Fadl, T. Hasegawa, A.F. Youssef, H.H. Farag, F.A. Omar, T. Kawaguchi, Synthesis and investigation of  $N^4$ -substituted cytarabine derivatives as prodrugs, *Pharmazie* 50 (1995) 382–387.
- [119] R.J. Ignoffo, Novel oral fluoropyrimidines in the treatment of metastatic colorectal cancer, *Am. J. Health-Syst. Pharm.* 56 (1999) 2417–2428.
- [120] Y.H. Choe, C.D. Conover, D. Wu, M. Royzen, R.B. Greenwald, Anticancer drug delivery systems:  $N^4$ -Acyl poly (ethylene glycol) prodrugs of Ara-C. I. Efficacy in solid tumors, *J. Control. Release* 79 (2002) 41–53.
- [121] D. Ranganathan, S. Kurur, Synthesis of totally chiral, multiple armed, poly glu and poly asp scaffoldings on a bifunctional adamantane core, *Tetrahedron Lett.* 38 (1997) 1265–1268.
- [122] Y.H. Choe, C.D. Conover, D. Wu, M. Royzen, Y. Gervacio, V. Borowski, M. Mehlig, R.B. Greenwald, Anticancer drug delivery systems: Multi-loaded  $N^4$ -Acyl poly (ethylene glycol) prodrugs of Ara-C. II. Efficacy in ascites and solid tumors, *J. Control. Release* 79 (2002) 55–70.
- [123] E. Nägele, M. Schelhaas, N. Kuder, H. Waldmann, Chemoenzymatic synthesis of N-Ras lipopeptides, *J. Am. Chem. Soc.* 120 (1998) 6889–6902.
- [124] S. Lee, R.B. Greenwald, J. McGuire, K. Yang, C. Shi, Drug delivery systems employing 1,6-elimination: releasable poly(ethylene glycol) conjugates of proteins, *Bioconjug. Chem.* 12 (2001) 163–169.
- [125] A. Abuchowski, T. van Es, N.C. Palczuk, F.F. Davis, Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol, *J. Biol. Chem.* 252 (1977) 3578–3581.
- [126] K. Yang, S. Lee, R.B. Greenwald, H. Zhao, C.D. Conover,

- H. Janjua, K.L. Shum, J. McGuire, Design and study of the linker controlled release of poly (ethylene glycol) conjugated protein, American Association of Pharmaceutical Scientists Conference: Pharmaceutics and Drug Delivery, April 22–24 (2002) Arlington, VA.
- [127] K.A. Witt, J.D. Huber, R.D. Egleton, M.J. Roberts, M.D. Bentley, L. Guo, H. Wei, H.I. Yamamura, T.P. David, Pharmacodynamic and pharmacokinetic characterization of poly(ethylene glycol) conjugation to met-enkephalin analog [D-PEG<sup>2</sup>, D-PEG<sup>5</sup>]-enkephalin (DPDPE), *J. Pharmacol. Exp. Ther.* 298 (2001) 848–856.
- [128] B.S. Lele, A.S. Hoffman, Mucoadhesive drug carriers based on complexes of poly(acrylic acid) and PEGylated drug having hydrolysable PEG-anhydride-drug linkages, *J. Control. Rel.* 69 (2000) 237–248.
- [129] R.B. Greenwald, Y.H. Choe, US Patent 5,965,119, October 12, 1999.
- [130] R.B. Greenwald, A. Pendri, Y.H. Choe, US Patent 6,180,095 B1, January 30, 2001.
- [131] S. Tsunoda, T. Ishikawa, Y. Yamamoto, H. Kamada, K. Koizumi, J. Matsui, Y. Tsutsumi, T. Hirano, T. Mayumi, Enhanced antitumor potency of polyethylene glycolylated tumor necrosis factor- $\alpha$ : a novel polymer-conjugation technique with a reversible amino-protective reagent, *J. Pharm. Exp. Ther.* 290 (1999) 368–372.
- [132] T. Suzawa, S. Nagamura, H. Saito, S. Ohta, N. Hanai, M. Yamasaki, Synthesis of a novel duocarmycin derivative DU-257 and its application to immunoconjugate using poly(ethylene glycol)-dipeptidyl linker capable of tumor specific activation, *Bioorg. Med. Chem.* 8 (2000) 2175–2184.
- [133] T. Suzawa, S. Nagamura, H. Saito, S. Ohta, N. Hanai, J. Kanazawa, M. Okabe, M. Yamasaki, Enhanced tumor cell selectivity of adriamycin-monoconal antibody conjugate via a poly (ethylene glycol)-based cleavable linker, *J. Controlled Release* 79 (2002) 229–242.

## Acid-Sensitive Polyethylene Glycol Conjugates of Doxorubicin: Preparation, In Vitro Efficacy and Intracellular Distribution

Paula C. A. Rodrigues,<sup>a</sup> Ulrich Beyer,<sup>a</sup> Peter Schumacher,<sup>a</sup> Thomas Roth,<sup>a</sup>  
Heinz H. Fiebig,<sup>a</sup> Clemens Unger,<sup>a</sup> Luigi Messori,<sup>b</sup> PierLuigi Orioli,<sup>b</sup>  
Dietrich H. Paper,<sup>c</sup> Rolf Mülhaupt<sup>d</sup> and Felix Kratz<sup>a,\*</sup>

<sup>a</sup>*Tumor Biology Center, Department of Medical Oncology, Clinical Research,  
Breisacher Straße 117, 79106 Freiburg, FRG*

<sup>b</sup>*Department of Chemistry, University of Florence, 51021 Florence, Italy*

<sup>c</sup>*Institute of Pharmacy, University of Regensburg, 93040 Regensburg, FRG*

<sup>d</sup>*Institute of Macromolecular Chemistry, University of Freiburg, 79104 Freiburg, FRG*

Received 17 February 1999; accepted 11 June 1999

**Abstract**—Coupling anticancer drugs to synthetic polymers is a promising approach of enhancing the antitumor efficacy and reducing the side-effects of these agents. Doxorubicin maleimide derivatives containing an amide or acid-sensitive hydrazone linker were therefore coupled to  $\alpha$ -methoxy-poly(ethylene glycol)-thiopropionic acid amide (MW 20000 Da),  $\alpha,\omega$ -bis-thiopropionic acid amide poly(ethylene glycol) (MW 20000 Da) or  $\alpha$ -*tert*-butoxy-poly(ethylene glycol)-thiopropionic acid amide (MW 70000 Da) and the resulting polyethylene glycol (PEG) conjugates isolated through size-exclusion chromatography. The polymer drug derivatives were designed as to release doxorubicin inside the tumor cell by acid-cleavage of the hydrazone bond after uptake of the conjugate by endocytosis. The acid-sensitive PEG conjugates containing the carboxylic hydrazone bonds exhibited in vitro activity against human BFX T24 bladder carcinoma and LXFL 529L lung cancer cells with IC<sub>70</sub> values in the range 0.02–1.5  $\mu$ m (cell culture assay: propidium iodide fluorescence or colony forming assay). In contrast, PEG doxorubicin conjugates containing an amide bond between the drug and the polymer showed no in vitro activity. Fluorescence microscopy studies in LXFL 529 lung cancer cells revealed that free doxorubicin accumulates in the cell nucleus whereas doxorubicin of the acid-sensitive PEG doxorubicin conjugates is primarily localized in the cytoplasm. Nevertheless, the acid-sensitive PEG doxorubicin conjugates retain their ability to bind to calf thymus DNA as shown by fluorescence and visible spectroscopy studies. Results regarding the effect of an acid-sensitive PEG conjugate of molecular weight 20000 in the chorioallantoic membrane (CAM) assay indicate that this conjugate is significantly less embryotoxic than free doxorubicin although antiangiogenic effects were not observed. © 1999 Elsevier Science Ltd. All rights reserved.

### Introduction

The attachment of antitumor drugs to synthetic polymers is a promising strategy of modifying their biodistribution, of reducing drug toxicity and thus improving the therapeutic efficacy of anticancer agents.<sup>1</sup> Polyethylene glycols (PEGs) are non-ionic, water-soluble synthetic polymers which are potential drug carriers due to their synthetic diversity and recognized biocompatibility.<sup>2</sup> In recent years, a number of reports have appeared on the synthesis and biological activity of high-molecular weight polyethylene glycol conjugates (MW

20–40 kDa) with antitumor agents such as paclitaxel and camptothecins.<sup>3,4</sup> These conjugates were designed to increase the water-solubility and plasma half-life of the drug while slowly releasing the parent compound through hydrolysis of the chemical link between the drug and the polymer backbone.

In our recent work on serum protein conjugates an acid-sensitive hydrazone linker was incorporated between the drug and the thiolated protein carrier allowing the protein-bound drug to be released in the acidic environment of endosomes and/or lysosomes after cellular uptake of the conjugate by endocytosis.<sup>5–9</sup> Acid-sensitive anthracycline and chlorambucil conjugates with serum albumin and transferrin exhibit high antiproliferative activity in vitro, and selected conjugates, such as acid-sensitive doxorubicin albumin conjugates, show superior antitumor

**Key words:** Doxorubicin; polyethylene glycol; drug polymer conjugates; acid-sensitivity; in vitro activity.

\* Corresponding author. Tel.: +49-761-2062176; fax: +49-761-2061899; e-mail: felix@tumorbio.uni-freiburg.de

efficacy in a number of animal tumor models when compared to the parent compound.<sup>10,11</sup>

As part of our research program on anticancer polymer conjugates we have now developed doxorubicin conjugates with polyethylene glycols of molecular weight 20000 and 70000 which contain an amide or an acid-sensitive hydrazone linker thus allowing a direct comparison with analogous transferrin and albumin doxorubicin conjugates. In this paper we report on the preparation of doxorubicin polyethylene glycol conjugates, on their antiproliferative activity in vitro and on the effect of selected conjugates in the chorioallantoic membrane (CAM) assay. In addition, we investigated the cellular uptake of the conjugates with fluorescence microscopy and followed their interaction with calf thymus DNA using UV/VIS- and fluorescence spectrophotometry. In this way we wanted to obtain a first insight into the biological activity and mechanistic

characteristics of the newly synthesized doxorubicin PEG conjugates.

## Results and Discussion

### Preparation and characterization

Polyethylene glycol conjugates of doxorubicin were prepared by reacting maleimide derivatives of doxorubicin (Hyd<sub>1</sub>, Hyd<sub>2</sub>, Amid<sub>1</sub>)<sup>12</sup> with  $\alpha$ -methoxy-poly(ethylene glycol)-thiopropionic acid amide (MW 20000 Da),  $\alpha,\omega$ -bis-thiopropionic acid amide poly(ethylene glycol) (MW 20000 Da) or  $\alpha$ -*tert*-butoxy-poly(ethylene glycol)-thiopropionic acid amide (MW 70000 Da) in aqueous media. The HS-group in the polymer adds to the double bond of the maleimide group in a fast and selective reaction forming a stable thioether bond. Subsequently, the resulting PEG doxorubicin conjugates

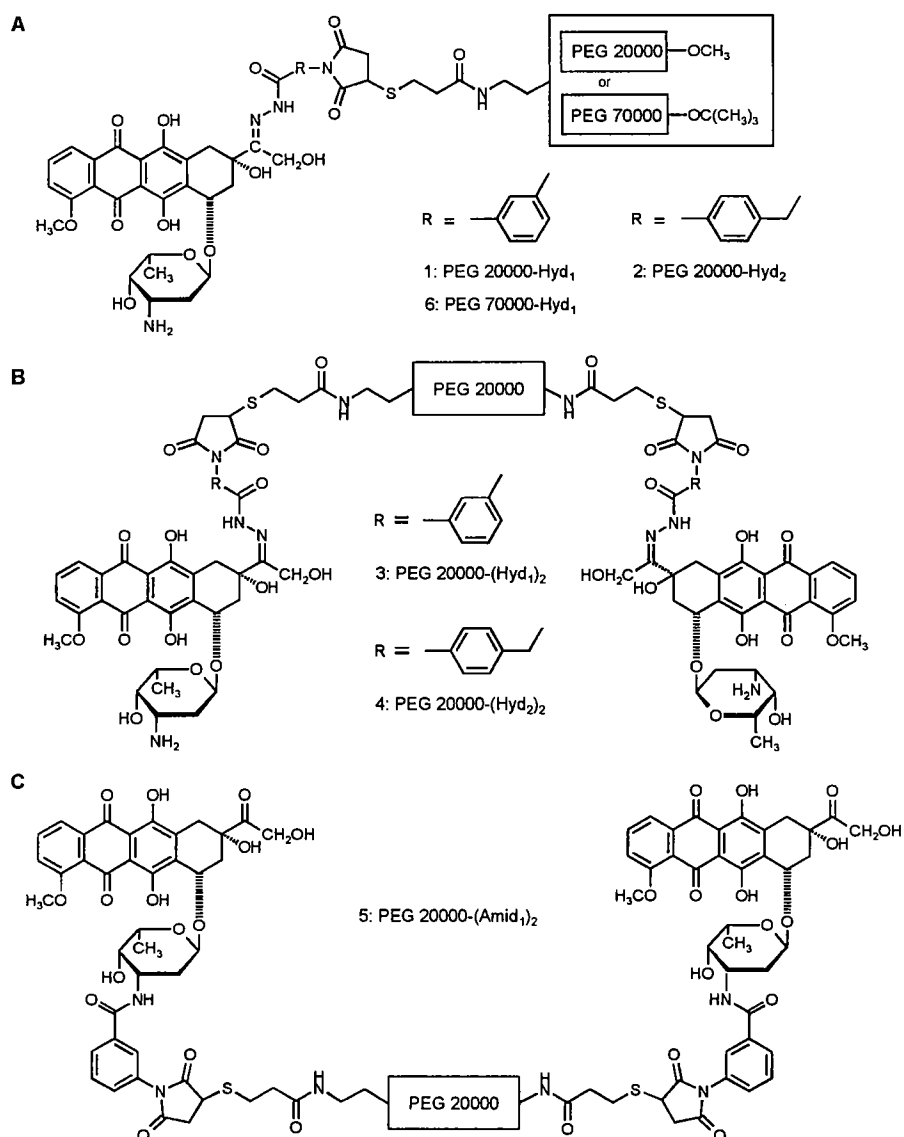


Figure 1. Structures of doxorubicin polyethylene glycol conjugates.

were isolated through size-exclusion chromatography over Sephadex® G-25 in phosphate buffer or over LH20® in methanol (see Experimental).

The purity of the samples was determined with an analytical HPLC-size exclusion column (Nucleogel® aqua-OH 40-8). A typical chromatogram, recorded at  $\lambda = 495$  nm, is shown in Figure 2. Retention times of the doxorubicin PEG conjugates are between 7 and 10 min on this column; free doxorubicin elutes as a broad peak at approximately 45 min. UV/VIS-spectra of the doxorubicin PEG conjugates in phosphate buffer showed the typical absorption maxima at  $\lambda = 495, 480, 252,$  and  $234$  nm. Selected vacuum-dried samples (**1** and **2**), which were obtained over Sephadex® LH20 in methanol, were investigated with  $^1\text{H}$  NMR spectroscopy in  $\text{CDCl}_3$  (400 and 600 MHz). Ethylene signals of the polyethylene glycol backbone were decoupled at 3.5 ppm. Analysis of the spectra revealed that distinct signals of the anthraquinone ring could be assigned, i.e. the HO-6 ( $\sim 14$  ppm, s) and HO-14 ( $\sim 13.3$  ppm, s) protons as well as the aromatic protons of ring A together with two proton signals of the spacer (7.5–8.0 ppm). In addition, the NH-proton of the carboxylic hydrazone bond showed a characteristic peak at  $\sim 10.5$  ppm. The typical strong singlet signal of the maleimide double bond ( $\sim 7.2$  ppm) was no longer present in the spectra indicating that the HS-group has reacted with the maleimide group. Assignment of the proton signals of the sugar ring of doxorubicin was not possible, however, due to a very broad signal of  $-\text{CH}_2$ -groups of the polymer despite our decoupling attempts.

### pH-Dependent stability studies

The three maleimide derivatives of doxorubicin (Hyd<sub>1</sub>, Hyd<sub>2</sub>, Amid<sub>1</sub>) differ in the site (3'-amino or 13-keto

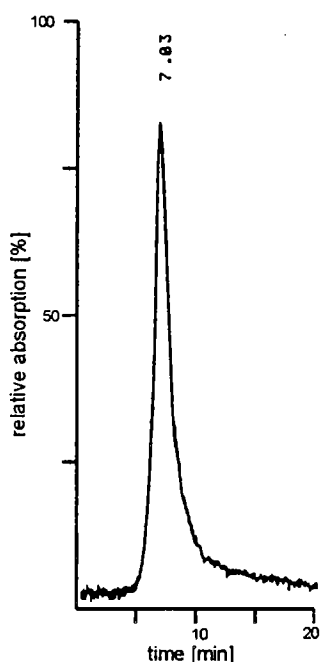


Figure 2. Chromatogram of PEG 20000-Hyd<sub>2</sub>; [flow = 1.0 ml/min.  $\lambda = 495$  nm,  $c = 30$   $\mu\text{M}$ ].

position) and stability (benzamide or benzoyl and phenylacetyl hydrazone bond) of the chemical link between doxorubicin and the spacer molecule. Previous stability studies demonstrated an acid-sensitive character of the carboxylic hydrazone bond.<sup>6,7</sup> In order to confirm this observation, pH-dependent stability of the doxorubicin PEG conjugates was studied at pH-values of 5.0 and 7.4 on our Nucleogel® column with the aid of HPLC. The decrease in the peak area of the conjugate recorded at  $\lambda = 495$  nm was used as a measure of doxorubicin release. The results of these studies are shown in Table 1.

Whereas the amide derivative **5** showed no release of doxorubicin at either pH 5.0 or 7.4, the carboxylic hydrazone derivatives **1–4** and **6** showed good stability at pH 7.4 (less than 10% release of doxorubicin after 48 h) but a marked release at pH 5.0. Acid-lability of the benzoyl hydrazone linker (Hyd<sub>1</sub>) was more pronounced in comparison to the phenylacetyl hydrazone linker (Hyd<sub>2</sub>). Furthermore, release of doxorubicin was slower for PEG 70000-Hyd<sub>1</sub> than for PEG 20000-Hyd<sub>1</sub> at pH 5.0.

### Biological data

The doxorubicin PEG conjugates and free doxorubicin were subsequently tested for biological activity in two human tumor cell lines (T24 bladder carcinoma and LXFL 529 lung cancer cells) using the propidium iodide fluorescence assay and in a human bladder carcinoma xenograft (BXF 1299) using a clonogenic assay. Respective IC<sub>70</sub> values are summarized in Table 2. Unbound polyethylene glycols had only marginal influence on cell growth in both cell lines (concentration range: 0.001–10  $\mu\text{M}$ ; data not shown). The conjugates **1** and **3** which contain a benzoyl hydrazone bond are more active than those containing a phenylacetyl hydrazone bond (**2** and **4**). In contrast, the amide conjugate **5** showed no activity at the concentrations tested (0.001–10  $\mu\text{M}$ ). PEG 70000-Hyd<sub>1</sub> was approximately 20-fold less active than PEG 20000-Hyd<sub>1</sub>. A comparison of the in vitro activity of the previously synthesized transferrin conjugates T-Doxo-Hyd<sub>1</sub> and T-Doxo-Hyd<sub>2</sub> with analogously constructed PEG doxorubicin conjugates in the BXF 1299 xenograft demonstrates that the IC<sub>70</sub> values lie in a very similar range (see Table 2). Of all compounds tested, free doxorubicin is the most active one exhibiting IC<sub>70</sub> values which are 5- to 200-fold lower than those for the acid-sensitive doxorubicin PEG conjugates.

Table 1. Stability studies of doxorubicin PEG conjugates at pH 5.0 and 7.4

Doxorubicin PEG conjugate	$t_{50}$ [h] <sup>a</sup> at pH 5.0	Stability at pH 7.4 <sup>b</sup>
<b>1</b> : PEG 20000-Hyd <sub>1</sub>	$\sim 2$	< 10%
<b>2</b> : PEG 20000-Hyd <sub>2</sub>	$\sim 30$	< 10%
<b>3</b> : PEG 20000-(Hyd <sub>1</sub> ) <sub>2</sub>	$\sim 2$	< 10%
<b>4</b> : PEG 20000-(Hyd <sub>2</sub> ) <sub>2</sub>	$\sim 6$	< 10%
<b>5</b> : PEG 20000-(Amid) <sub>2</sub>	> 72	< 5%
<b>6</b> : PEG 70000-Hyd <sub>1</sub>	$\sim 27$	< 10%

<sup>a</sup> Time after which the peak area of the conjugate is 50% of its initial value.

<sup>b</sup> Decrease in peak area of the conjugate after 48h.



**Table 2.** In vitro antitumor activity of doxorubicin, of PEG and transferrin doxorubicin conjugates in two human tumor cell lines (T24, LXFL 529) and in the BXF 1299 xenograft

Compound	IC <sub>70</sub> value in T24 [ $\mu$ M] <sup>a</sup>	IC <sub>70</sub> value in LXFL 529 [ $\mu$ M] <sup>a</sup>	IC <sub>70</sub> value in BXF 1299 [ $\mu$ M] <sup>b</sup>
Doxorubicin	0.004	<0.001	0.15
1: PEG 20000-Hyd <sub>1</sub>	0.03	0.04	0.30
2: PEG 20000-Hyd <sub>2</sub>	0.34	0.09	0.50
3: PEG 20000-(Hyd <sub>1</sub> ) <sub>2</sub>	0.06	0.02	0.25
4: PEG 20000-(Hyd <sub>2</sub> ) <sub>2</sub>	0.39	0.26	1.50
5: PEG 20000-(Amid) <sub>2</sub>	> 10	> 10	Not determined
6: PEG 70000-Hyd <sub>1</sub>	0.60	0.12	Not determined
T-Doxo-Hyd <sub>1</sub>	Not determined	Not determined	0.5
T-Doxo-Hyd <sub>2</sub>	Not determined	Not determined	1.8

<sup>a</sup> Propidium iodide fluorescence assay: the fluorescence assay was performed according to the method Dengler et al.<sup>17</sup> (also described by us in 6 and 8). After 6 days of continuous drug exposure nonviable cells were stained by addition of propidium iodide, and fluorescence (FU<sub>1</sub>) was measured (excitation 530 nm, emission 620 nm). Microplates were then kept at –18°C for 24 h, which resulted in total cell kill. After thawing of the plates a second fluorescence measurement (FU<sub>2</sub>) was carried out, and the amount of viable cells was calculated by FU<sub>2</sub>/FU<sub>1</sub>.

<sup>b</sup> Clonogenic assay: the assay was performed as a two-layer soft agar assay using a cell suspension derived from the human tumor xenograft BXF 1299 as described in detail in Fiebig et al.<sup>18</sup> Drug effects were evaluated after 15 days of drug exposure.

### CAM-assay<sup>13,14</sup>

Doxorubicin and PEG 20000-Hyd<sub>2</sub> were tested in the CAM-assay for their antiangiogenic effect compared to standard controls (laminarin sulphate, suramin), see Table 3. At the concentration of 20  $\mu$ g/pellet for free doxorubicin all embryos died within one day (data not shown) so that the maximum drug concentration of 10  $\mu$ g/pellet was used in the experiments. PEG 20000-Hyd<sub>2</sub> is less toxic because it did not show any embryotoxic effect at the concentration of 10  $\mu$ g doxorubicin equivalents/pellet. Free doxorubicin exhibited a very low antiangiogenic effect (score 0.4), and PEG 20000-Hyd<sub>2</sub> is inactive according to the score rating in the CAM assay. In conclusion, the data indicates that PEG 20000-Hyd<sub>2</sub> is better tolerated than free doxorubicin.

### Fluorescence microscopy studies

In order to investigate the cellular uptake and intracellular distribution of doxorubicin and the acid-sensitive doxorubicin PEG conjugates, LXFL 529 cells were incubated with our drug formulations for 24 h and doxorubicin was subsequently detected by fluorescence

microscopy. Fluorescence microscopy is a suitable technique to study the intracellular fate of the auto-fluorescent drug doxorubicin and to assess any qualitative differences between anthracycline derivatives regarding their intracellular distribution. Results are depicted in Figure 3 for doxorubicin and PEG 20000-Hyd<sub>2</sub> as a representative example. As shown in Figure 3A, doxorubicin is confined to the cell nucleus after 24 h. In contrast, when cells were incubated with PEG 20000-Hyd<sub>2</sub> for 24 h, decreased fluorescence was detected in the nucleus but was observed primarily in the cytoplasm (Fig. 3B). PEG 70000-Hyd<sub>1</sub> displayed a similar cellular distribution pattern (data not shown). The differences in the intracellular distribution are in accordance with our earlier work on acid-sensitive transferrin-doxorubicin conjugates in this cell line,<sup>6</sup> in which LXFL 529 cells treated with the acid-sensitive transferrin-doxorubicin conjugate (T-Doxo-Hyd<sub>1</sub>) were devoid of nuclear fluorescence after 24 h.

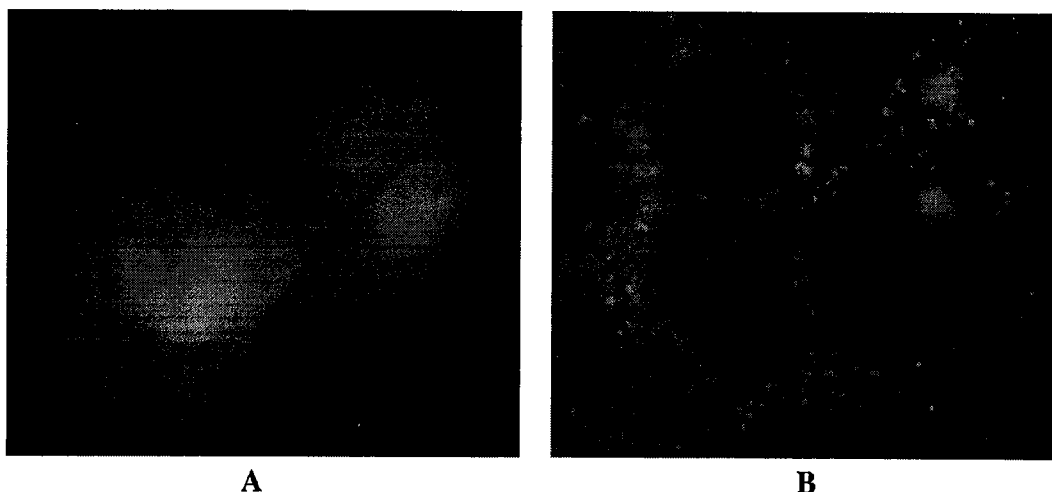
### Interactions with calf thymus DNA

In light of the results from our fluorescence microscopy studies it was of interest to establish whether the anthracycline chromophore of the doxorubicin PEG conjugates retains its ability to bind DNA through intercalation. Free doxorubicin and the conjugates **1**, **2** and **5** were therefore reacted with calf thymus DNA under physiological conditions and the reaction analyzed by fluorescence spectroscopy and visible spectrophotometry. It is well known that DNA-intercalation of doxorubicin results in a dramatic quenching (>90%) of the intrinsic fluorescence of the anthracycline chromophore.<sup>15,16</sup> A similar fluorescence quenching was observed when **1** (~90% quenching) or **2** (~75% quenching) was incubated with saturating amounts of calf thymus DNA as shown in Figure 4(B and C) (Fig. 4A shows the results for free doxorubicin under the same conditions, quenching >90%). In contrast, quenching is significantly less marked in the case of **5**, which is the in vitro inactive amide conjugate (quenching ~20%, see Fig. 4D), and implies that DNA-intercalation is less efficient. These results were confirmed by

**Table 3.** Antiangiogenic and embryotoxic effects of doxorubicin, PEG 20000-Hyd<sub>2</sub>, suramin, laminarin sulphate, and agarose in the CAM-assay

Compound	Antiangiogenic effect (score-value)	Embryotoxic effect (loss of embryos in %)
Doxorubicin <sup>a</sup>	0.4 ( $\pm$ 0.15)	31%
PEG 20000-Hyd <sub>2</sub> <sup>b</sup>	0	No effect
Suramin <sup>c</sup>	0.5 $\pm$ 0.2	No effect
laminarin sulphate <sup>a</sup>	1.05 ( $\pm$ 0.05)	No effect
Agarose	0	No effect

Test concentrations: <sup>a</sup> 10  $\mu$ g/pellet; <sup>b</sup> 10  $\mu$ g doxorubicin equivalents/pellet; <sup>c</sup> 50  $\mu$ g/pellet. Score values: 0: no effect; 0.5: very weak effect, no capillary free area or area with a reduced density of capillaries around the pellet not larger than the area of the pellet; 1: medium effect, small capillary free area or area with significantly reduced density of capillaries, effects not larger than twice the size of the pellet. 2: strong effect, capillary free area around the pellet at least twice the size of the pellet.



**Figure 3.** Fluorescence microscopy photographs of the cellular distribution of doxorubicin (A) and PEG 20000-Hyd<sub>2</sub> (B) after 24 h; LXFL 529 lung cancer cells were incubated with drug concentrations of  $c \approx 1 \mu\text{M}$  for 24 h. Fluorescence was excited at 450–490 nm and detected at a wavelength greater than 540 nm.

spectrophotometry in the visible region demonstrating that reaction of doxorubicin, **1** or **2** with DNA produced a marked change in the initial spectra without DNA which consisted in a considerable decrease and red shift of the main visible band (450–550 nm), data not shown. Smaller spectral perturbations were observed for **5**.

### Summary

The purpose of the present study was to obtain a first picture of the biological activity and mode of action of doxorubicin PEG conjugates. From our data we conclude that the acid-sensitive properties of the link between PEG and doxorubicin are important for retaining the antitumor activity of doxorubicin. In principle, the order of antitumor activity of the conjugates correlates with their acid-lability. Fluorescence microscopy studies in LXFL 529 lung cancer cells show that free doxorubicin accumulates in the cell nucleus whereas doxorubicin of the acid-sensitive PEG conjugates is primarily localized in the cytoplasm. These results indicate that the conjugates might exert their cytotoxicity by a different mode of action other than intercalation with DNA. However, the acid-sensitive PEG doxorubicin conjugates retain their ability to bind to calf thymus DNA as shown by fluorescence and visible spectroscopy studies. Thus, in order to understand the mode of action of acid-sensitive anthracycline PEG conjugates in more detail, we are at present carrying out laser scanning confocal fluorescence microscopy studies as well as incubation studies with RNA.

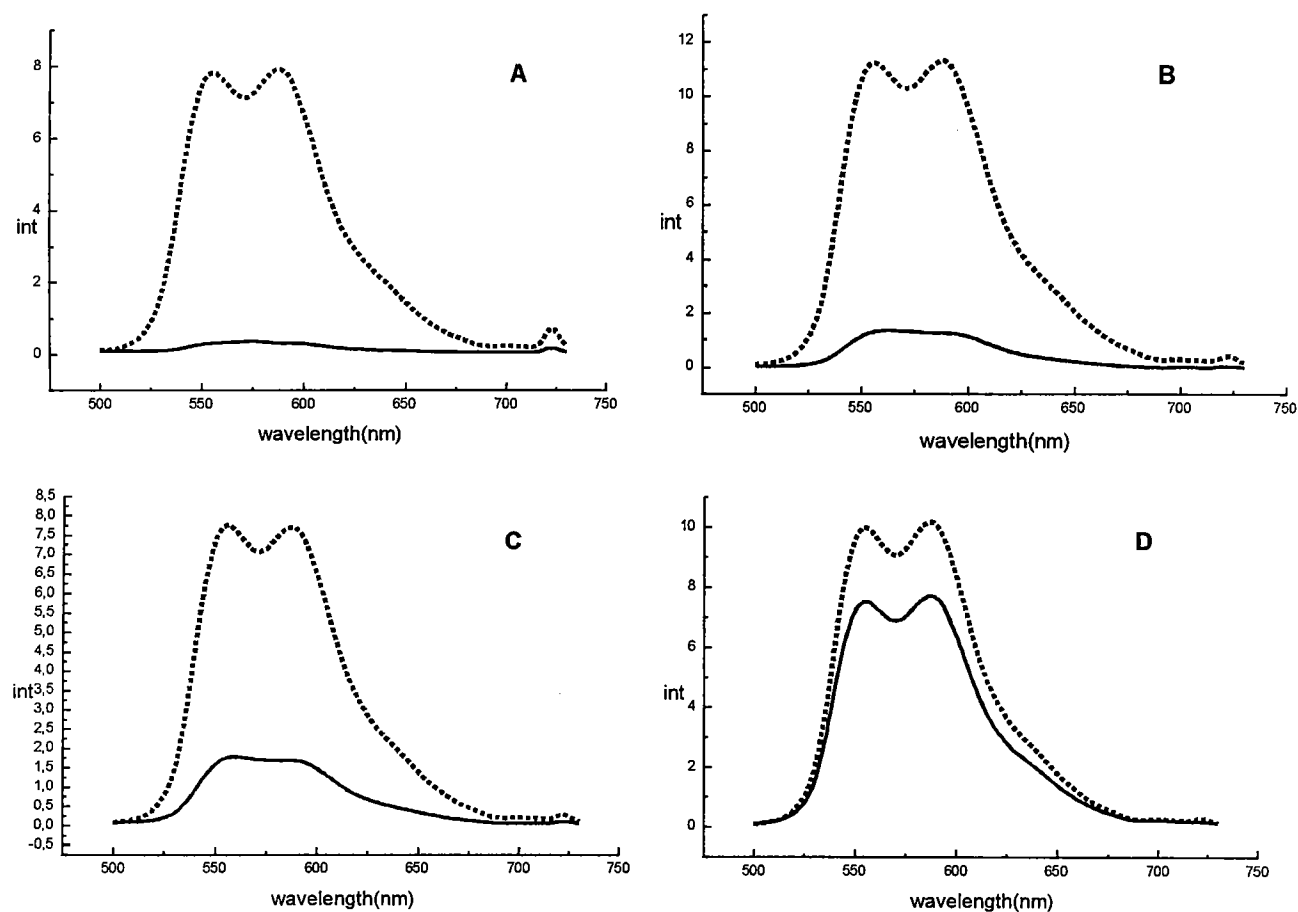
Although the IC<sub>70</sub> values of the conjugates are higher than for free doxorubicin, they nonetheless lie in a range which is relevant for further preclinical evaluation. From our experience, the benefit of antitumor macromolecular prodrugs can primarily be proven in suitable

animal models due to the great difference in the biodistribution between the free drug and the drug polymer conjugate.<sup>10,11</sup> Our preliminary data in nude mice show that PEG 20000-(Hyd<sub>2</sub>)<sub>2</sub> (**3**) and PEG 70000-Hyd<sub>1</sub> (**6**) can be administered at far higher doses than the maximum tolerated dose of free doxorubicin ( $2 \times 16$ – $24 \text{ mg/kg}$  as compared to  $2 \times 8 \text{ mg/kg}$ ). This is in accordance with our experience with analogous transferrin and albumin conjugates of doxorubicin.<sup>6,7,10,11</sup> Our data with PEG 20000-Hyd<sub>2</sub> in the CAM assay show that this conjugate is significantly less embryotoxic than free doxorubicin although antiangiogenic effects were not observed. In vivo experiments evaluating the antitumor efficacy of acid-sensitive doxorubicin PEG conjugates in xenograft models are in progress.

### Experimental

#### Chemicals, materials and spectroscopy

<sup>1</sup>H NMR and <sup>13</sup>C NMR: Bruker 400 or 600 MHz AMX (internal standard: TMS); Analytical HPLC: HPLC studies were performed on an analytical HPLC column (Nucleogel® aqua-OH 40-8, 300 mm  $\times$  7.7 mm, from Macherey&Nagel, FRG); mobile phase: 0.15 M NaCl, 0.01 M sodium phosphate, 10% v/v CH<sub>3</sub>CN, 30% v/v MeOH-pH 7.0. A Lambda 1000 UV-visible monitor from Bischoff (at  $\lambda = 495 \text{ nm}$ ), an autosampler Merck Hitachi AS400 and an Integrator Merck Hitachi D2500 were used; doxorubicin was a gift from Pharmacia and Upjohn, FRG. Organic solvents: HPLC grade (Merck). Other organic or inorganic compounds: Merck AG, FRG. Hyd<sub>1</sub>, Hyd<sub>2</sub>, Amid<sub>1</sub> were prepared previously.<sup>12</sup> PEGs were purchased from Rapp Polymer, FRG; the buffers used were vacuum-filtered through a 0.2  $\mu\text{m}$  membrane (Sartorius, FRG). Cell culture media, supplements (L-glutamine, antibiotics, trypsin versene/EDTA) and fetal calf serum (FCS) were purchased from



**Figure 4.** Fluorescence spectra of doxorubicin (A), of **2** (B), **1** (C) and **5** (D) ( $c \approx 1 \mu\text{M}$ ) in the absence (---) and in the presence (—) of 0.6 mg/mL calf thymus DNA.

Bio Whittaker (Serva, Heidelberg, FRG). Propidium iodide was purchased from Aldrich-Sigma-Chemie, FRG. All culture flasks were obtained from Greiner Labortechnik (Frickenhausen, FRG).

**Methods for the preparation of polyethylene glycol conjugates of doxorubicin.** FPLC for preparation of conjugates: P-500 pump, LCC 501 Controller (Pharmacia) and LKB 2151 UV-monitor (at  $\lambda = 280 \text{ nm}$ ); buffer: 0.004 M sodium phosphate, 0.15 M NaCl, pH 7.4. All reactions were performed at room temperature unless otherwise stated. Data for one representative experiment is given:

**Preparation of PEG 20000-(Hyd<sub>1</sub>)<sub>2</sub>.** Eight milligrams (0.01 mmol) of Hyd<sub>1</sub> were dissolved in 250  $\mu\text{L}$  dimethylformamide and added to 50 mg (0.0025 mmol) PEG-20000(SH)<sub>2</sub> dissolved in 5 mL buffer (0.004 M sodium phosphate, 0.15 mol NaCl, pH 6.8). The mixture was homogenized and kept at room temperature for 30 min. After centrifuging the slightly turbid mixture for 5 min with a Sigma 112 centrifuge, the supernatant was loaded on a Sephadex<sup>®</sup> G 25 column (100 mm  $\times$  20 mm, loop size: 5 mL). The conjugate eluted with a retention time of 5–10 min (flow: 1.0 mL/min, buffer: 0.004 M sodium phosphate, 0.15 M NaCl, pH 7.4). Concentration of the conjugate to a volume of approximately 2 mL was carried out with CENTRIPREP<sup>®</sup>-10-concentrators from

Amicon, FRG (60 min at 4°C and 4500 rpm). The concentration of doxorubicin in the conjugate was adjusted to  $c = 300 \pm 20 \mu\text{M}$  using the  $\epsilon$ -value for doxorubicin in physiological buffer ( $\epsilon_{495} = 10650 \text{ M}^{-1} \text{ cm}^{-1}$ ).<sup>6,7</sup> The conjugate was stored at  $-80^\circ\text{C}$ . Doxorubicin polyethylene glycol conjugates for NMR studies were chromatographed over Sephadex<sup>®</sup> LH20 Gel (100  $\times$  10 mm, loop size: 2 mL, flow: 1.0 mL/min, retention time: 3–6 min, eluent: 100% methanol HPLC grade).

**pH dependent stability studies with the PEG doxorubicin conjugates at pH 5.0 and 7.4.** Fifty microlitres of the stock solutions of the conjugates ( $c = 300 \pm 20 \mu\text{M}$ ) in phosphate buffer were added to 450  $\mu\text{L}$  of buffer pH 5.0 (0.15 M NaCl, 0.004 M sodium phosphate adjusted to pH 5.0 with hydrochloric acid) or pH 7.4 (0.15 M NaCl, 0.004 M sodium phosphate). The solutions were incubated at room temperature and 50  $\mu\text{L}$  samples were analyzed at  $\lambda = 495 \text{ nm}$  every 2–4 h over a period of 72 h on an analytical HPLC column (Nucleogel<sup>®</sup> aqua-OH 40-8, 300  $\times$  7.7 mm, from Macherey & Nagel, FRG); mobile phase: 0.15 M NaCl, 0.01 M sodium phosphate, 10% v/v CH<sub>3</sub>CN, 30% v/v MeOH-pH 7.0.

**Fluorescence spectra.** Fluorescence spectra were carried out with a Jasco FP-750 spectrofluorimeter working at room temperature. Fluorescence was excited at 480 nm

and detected at wavelengths greater than 500 nm. Doxorubicin or conjugates were diluted with buffer (8 mM phosphate, 185 mM NaCl, 1 mM EDTA, pH 7.4) to a concentration of 1.0  $\mu$ M. Calf thymus DNA (Sigma) was then added to a final concentration of  $r \sim 0.01$ , where  $r$  is the ratio between the molar concentration of doxorubicin and the DNA basepairs. Fluorescence spectra were recorded after incubating the samples for a few minutes.

**Fluorescence microscopy.** LXFL 529 lung carcinoma cells were grown in RPMI 1640 cell culture medium with 10% FCS to which were added 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin and 100  $\mu$ g/mL L-glutamine. After subcultivation, cells were washed twice in PBS, resuspended in medium to a final concentration of  $2 \times 10^4$  cells/cm<sup>2</sup> and allowed to adhere on sterile TC chamber slides with glass bottom (NUNC, Denmark) for 24 h. Cells were incubated with drug concentrations of  $\approx 1 \mu$ M for 24 h. Slides were then washed twice in PBS and covered with 24  $\times$  50 mm coverslips. Cell preparations were studied by epi-illumination with the use of interference optics. An Olympus microscope fitted with an HBO-100 mercury-arc lamp was used. The microscope contained a 2-type filter complex for fluorescein detection that consisted of a 450–490 nm excitation filter, a 510 nm dichroic mirror, and a 540 nm barrier filter that allows wavelength greater than 540 nm to pass. The microscope was connected to an Olympus camera containing a 35 mm ASA 400 Elitechrome film. Fluorescence microscopy studies were repeated twice for doxorubicin and each conjugate.

**Biology.** Human tumor cells were grown at 37°C in a humidified atmosphere (95% air, 5% CO<sub>2</sub>) in monolayer RPMI 1640 culture medium with phenol red supplemented with 10% heat inactivated FCS, 300 mg/L glutamine and 1% antibiotic solution (5,000  $\mu$ g gentamycin/mL). Cells were trypsinized and maintained twice a week. The concentration of free doxorubicin in the stock solution of the conjugates was 300  $\mu$ M.

**Propidium iodide fluorescence assay.** The fluorescence assay was performed according to the method of Dengler et al.<sup>17</sup> Briefly, cells were harvested from exponential phase cultures growing in RPMI culture medium by trypsinization, counted and plated in 96-well flat-bottomed microtitre plates (50  $\mu$ L cell suspension/well,  $1.0 \times 10^5$  cells/mL). After a 24 h recovery in order to allow cells to resume exponential growth, 100  $\mu$ L culture medium (6 control wells per plate) or culture medium containing drug was added to the wells. Each drug concentration was plated in triplicate. After 6 days of continuous drug exposure nonviable cells were stained by addition of 25  $\mu$ L of a propidium iodide solution (50  $\mu$ g/mL). Fluorescence (FU<sub>1</sub>) was measured using a Millipore Cytofluor 2350 microplate reader (excitation 530 nm, emission 620 nm). Microplates were then kept at –18°C for 24 h, which resulted in a total cell kill. After thawing of the plates and a second fluorescence measurement (FU<sub>2</sub>) the amount of viable cells was calculated by FU<sub>2</sub>/FU<sub>1</sub>. Growth inhibition was expressed as treated/control  $\times 100$  (%T/C).

**Clonogenic assay.** The assay was performed as a two-layer soft agar assay using a cell suspension derived from the human tumor xenograft BXF 1299 as described in detail in Fiebig et al.<sup>18</sup>

**In vivo test for antiangiogenic activity.** Test compounds were dissolved in a 2.5% agarose-solution (final concentrations: 1–5 mg/mL) at approximately 60°C. For preparing the pellets 10  $\mu$ L of the respective warm solution were dropped on circular teflon supports of 3 mm in diameter and then cooled instantly to room temperature. The fertilized hen eggs were incubated for 65–70 h at 37°C at a relative humidity of 80%, positioned horizontally and rotated several times before being opened on the snub side. Prior to this 10 mL of albumin were aspirated from a hole on the pointed side. At two thirds of the height (from the pointed side) the eggs were traced with a scalpel and the shells removed with forceps. The aperture was covered with keep-fresh film, and the eggs were incubated at 37°C at a relative humidity of 80% for 75 h. When the formed chorioallantoic membrane had reached a diameter of approximately 2 cm, one pellet per egg was placed on it. The eggs were incubated for one further day and then evaluated under the stereo microscope. For every test compound 15–20 eggs were used. Each experiment was performed in duplicate (for details see refs 13 and 14).

#### Acknowledgements

This work has been supported by NATO/INTAS funds, the Dr. Mildred Scheel Stiftung der Deutschen Krebshilfe, FRG, the Fördergesellschaft der Klinik für Tumorbologie and a grant by the Fonds der Chemischen Industrie (BMBF). We thank Pharmacia & Upjohn for providing us with doxorubicin.

#### References

1. Maeda, H.; Matsumura, Y. *Crit. Rev. Ther. Drug Carrier Sys.* **1989**, 6, 193.
2. Zalipsky, S.; Gilon, C.; Zilicha, A. *Eur. Polym. J.* **1983**, 19, 1177.
3. Greenwald, R. B.; Gilbert, C. W.; Pedri, A.; Conover, C. D.; Xia, J.; Martinez, A. *J. Med. Chem.* **1996**, 39, 424.
4. Greenwald, R. B.; Pendri, A.; Conover, C. D.; Choe, Y. H.; Gilbert, C. W.; Martinez, A.; Xia, J.; Wu, H.; Hsue, M. *Bioorg. Med. Chem.* **1998**, 6, 551.
5. Kratz, F.; Beyer, U.; Schumacher, P.; Krüger, M.; Zahn, H.; Roth, T.; Fiebig, H. H.; Unger, C. *Bioorg. Med. Chem. Lett.* **1997**, 7, 617.
6. Kratz, F.; Beyer, U.; Roth, T.; Tarasova, N.; Collery, P.; Lechenault, F.; Cazabat, A.; Schumacher, P.; Unger, C.; Falken, U. *J. Pharm. Sci.* **1998**, 87, 338.
7. Kratz, F.; Beyer, U.; Collery, P.; Lechenault, F.; Cazabat, A.; Schumacher, P.; Falken, U.; Unger, C. *Biol. Pharm. Bull.* **1998**, 21, 56.
8. Kratz, F.; Beyer, U.; Roth, T.; Schütte, M. T.; Unold, A.; Fiebig, H. H.; Unger, C. *Arch. Pharm. Med. Chem.* **1998**, 331, 47.
9. Beyer, U.; Roth, T.; Schumacher, P.; Maier, G.; Unold, A.; Frahm, A. W.; Fiebig, H. H.; Unger, C.; Kratz, F. *J. Med. Chem.* **1998**, 41, 2701.

10. Kratz, F.; Fichtner, I.; Beyer, U.; Schumacher, P.; Roth, T.; Fiebig, H. H.; Unger, C. *Eur. J. Cancer* **1997**, *33*, S175.
11. Dreves, J.; Hofmann, I.; Marmé, D.; Unger, C.; Kratz, F. *Drug Delivery* **1999**, *6*, 1.
12. Krüger, M.; Beyer, U.; Zahn, H.; Unger, C.; Kratz, F. *Chem. Pharm. Bull.* **1997**, *45*, 399.
13. Paper, D. H.; Vogl, H.; Franz, G.; Hoffman, R. *Macromol. Symp.* **1995**, *99*, 219.
14. Hoffman, R.; Paper, D. H.; Donaldson, J.; Vogl, H. *Br. J. Cancer* **1996**, *73*, 1183.
15. Chaires, J. B.; Dattagupta, N.; Crothers, D. M. *Biochemistry* **1982**, *21*, 3933.
16. Barabas, K.; Sizensky, J. A.; Faulk, W. P. *J. Biol. Chem.* **1992**, *267*, 9437.
17. Dengler, W. A.; Schulte, J.; Berger, D. P.; Mertelsmann, R.; Fiebig, H. H. *Anti-Cancer Drugs* **1995**, *6*, 522.
18. Drees, M.; Dengler, W. A.; Roth, T.; Labonte, H.; Mayo, J.; Malspeis, L.; Grever, M.; Sausville, E. A.; Fiebig, H. H. *Clin. Cancer Res.* **1997**, *13*, 273.

### **AVONEX® (Interferon beta-1a)**

#### **IM Injection**

#### **DESCRIPTION**

AVONEX® (Interferon beta-1a) is a 166 amino acid glycoprotein with a predicted molecular weight of approximately 22,500 daltons. It is produced by recombinant DNA technology using genetically engineered Chinese Hamster Ovary cells into which the human interferon beta gene has been introduced. The amino acid sequence of AVONEX® is identical to that of natural human interferon beta.

Using the World Health Organization (WHO) natural interferon beta standard, Second International Standard for Interferon, Human Fibroblast (Gb-23-902-531), AVONEX® has a specific activity of approximately 200 million international units (IU) of antiviral activity per mg of Interferon beta-1a determined specifically by an *in vitro* cytopathic effect bioassay using lung carcinoma cells (A549) and Encephalomyocarditis virus (ECM). AVONEX® 30 mcg contains approximately 6 million IU of antiviral activity using this method. The activity against other standards is not known. Comparison of the activity of AVONEX® with other Interferon betas is not appropriate, because of differences in the reference standards and assays used to measure activity.

#### 30 mcg Lyophilized Powder Vial

A vial of AVONEX® is formulated as a sterile, white to off-white lyophilized powder for intramuscular injection after reconstitution with supplied diluent (Sterile Water for Injection, USP). Each vial of reconstituted AVONEX® contains 30 mcg of Interferon beta-1a; 15 mg Albumin (Human), USP; 5.8 mg Sodium Chloride, USP; 5.7 mg Dibasic Sodium Phosphate, USP; and 1.2 mg Monobasic Sodium Phosphate, USP, in 1.0 mL at a pH of approximately 7.3.

#### 30 mcg Prefilled Syringe

A prefilled syringe of AVONEX® is formulated as a sterile liquid for intramuscular injection. Each 0.5 mL (30 mcg dose) of AVONEX® in a prefilled glass syringe contains 30 mcg of Interferon beta-1a, 0.79 mg Sodium Acetate Trihydrate, USP; 0.25 mg Glacial Acetic Acid,

USP; 15.8 mg Arginine Hydrochloride, USP; and 0.025 mg Polysorbate 20 in Water for Injection, USP at a pH of approximately 4.8.

## **CLINICAL PHARMACOLOGY**

### **General**

Interferons are a family of naturally occurring proteins and glycoproteins that are produced by eukaryotic cells in response to viral infection and other biological inducers. Interferon beta, one member of this family, is produced by various cell types including fibroblasts and macrophages. Natural interferon beta and Interferon beta-1a are glycosylated, with each containing a single N-linked complex carbohydrate moiety. Glycosylation of other proteins is known to affect their stability, activity, aggregation, biodistribution, and half-life in blood. However, the effects of glycosylation of interferon beta on these properties have not been fully defined.

### **Biologic Activities**

Interferons are cytokines that mediate antiviral, antiproliferative and immunomodulatory activities in response to viral infection and other biological inducers. Three major interferons have been distinguished: alpha, beta, and gamma. Interferons alpha and beta form the Type I class of interferons, and interferon gamma is a Type II interferon. These interferons have overlapping but clearly distinct biological activities.

Interferon beta exerts its biological effects by binding to specific receptors on the surface of human cells. This binding initiates a complex cascade of intracellular events that leads to the expression of numerous interferon-induced gene products and markers. These include 2', 5'-oligoadenylate synthetase,  $\beta_2$ -microglobulin, and neopterin. These products have been measured in the serum and cellular fractions of blood collected from patients treated with AVONEX®.

The specific interferon-induced proteins and mechanisms by which AVONEX® exerts its effects in multiple sclerosis have not been fully defined. Clinical studies conducted in multiple sclerosis patients showed that interleukin 10 (IL-10) levels in cerebrospinal fluid were increased in patients treated with AVONEX® compared to placebo. Serum IL-10 levels were increased 48 hours after intramuscular (IM) injection of AVONEX® and remained

elevated for 1 week. However, no relationship has been established between absolute levels of IL-10 and clinical outcome in multiple sclerosis.

### **Pharmacokinetics**

Pharmacokinetics of AVONEX® in multiple sclerosis patients have not been evaluated. The pharmacokinetic and pharmacodynamic profiles of AVONEX® in healthy subjects following doses of 30 mcg through 75 mcg have been investigated. Serum levels of AVONEX® as measured by antiviral activity are slightly above detectable limits following a 30 mcg IM dose, and increase with higher doses.

After an IM dose, serum levels of AVONEX® typically peak between 3 and 15 hours and then decline at a rate consistent with a 10 hour elimination half-life. Serum levels of AVONEX® may be sustained after IM administration due to prolonged absorption from the IM site. Systemic exposure, as determined by AUC and C<sub>max</sub> values, is greater following IM than subcutaneous (SC) administration.

Subcutaneous administration of AVONEX® should not be substituted for intramuscular administration. Subcutaneous and intramuscular administration have been observed to have non-equivalent pharmacokinetic and pharmacodynamic parameters following administration to healthy volunteers.

Biological response markers (e.g., neopterin and β<sub>2</sub>-microglobulin) are induced by AVONEX® following parenteral doses of 15 mcg through 75 mcg in healthy subjects and treated patients. Biological response marker levels increase within 12 hours of dosing and remain elevated for at least 4 days. Peak biological response marker levels are typically observed 48 hours after dosing. The relationship of serum AVONEX® levels or levels of these induced biological response markers to the mechanisms by which AVONEX® exerts its effects in multiple sclerosis is unknown.

### **Clinical Studies**

The clinical effects of AVONEX® in multiple sclerosis were studied in two randomized, multicenter, double-blind, placebo-controlled studies in patients with multiple sclerosis.<sup>1,2</sup> Safety and efficacy of treatment with AVONEX® beyond 3 years is not known.



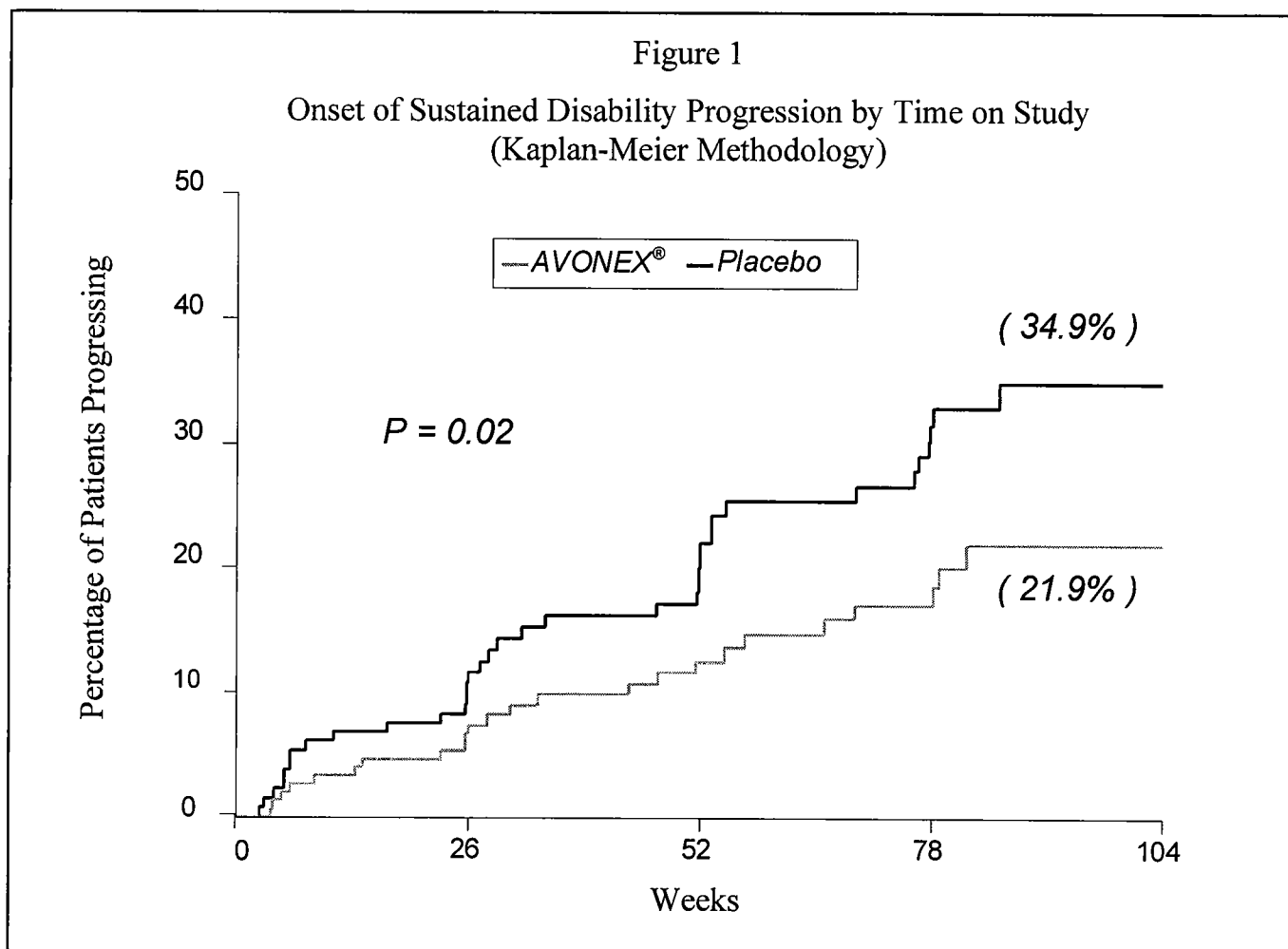
In Study 1, 301 patients received either 30 mcg of AVONEX® (n=158) or placebo (n=143) by IM injection once weekly. Patients were entered into the trial over a 2½ year period, received injections for up to 2 years, and continued to be followed until study completion. Two hundred eighty-two patients completed 1 year on study, and 172 patients completed 2 years on study. There were 144 patients treated with AVONEX® for more than 1 year, 115 patients for more than 18 months and 82 patients for 2 years.

All patients had a definite diagnosis of multiple sclerosis of at least 1 year duration and had at least 2 exacerbations in the 3 years prior to study entry (or 1 per year if the duration of disease was less than 3 years). At entry, study participants were without exacerbation during the prior 2 months and had Kurtzke Expanded Disability Status Scale (EDSS<sup>3</sup>) scores ranging from 1.0 to 3.5. Patients with chronic progressive multiple sclerosis were excluded from this study.

The primary outcome assessment was time to progression in disability, measured as an increase in the EDSS score of at least 1.0 point that was sustained for at least 6 months. An increase in EDSS score reflects accumulation of disability. This endpoint was used to ensure that progression reflected permanent increase in disability rather than a transient effect due to an exacerbation.

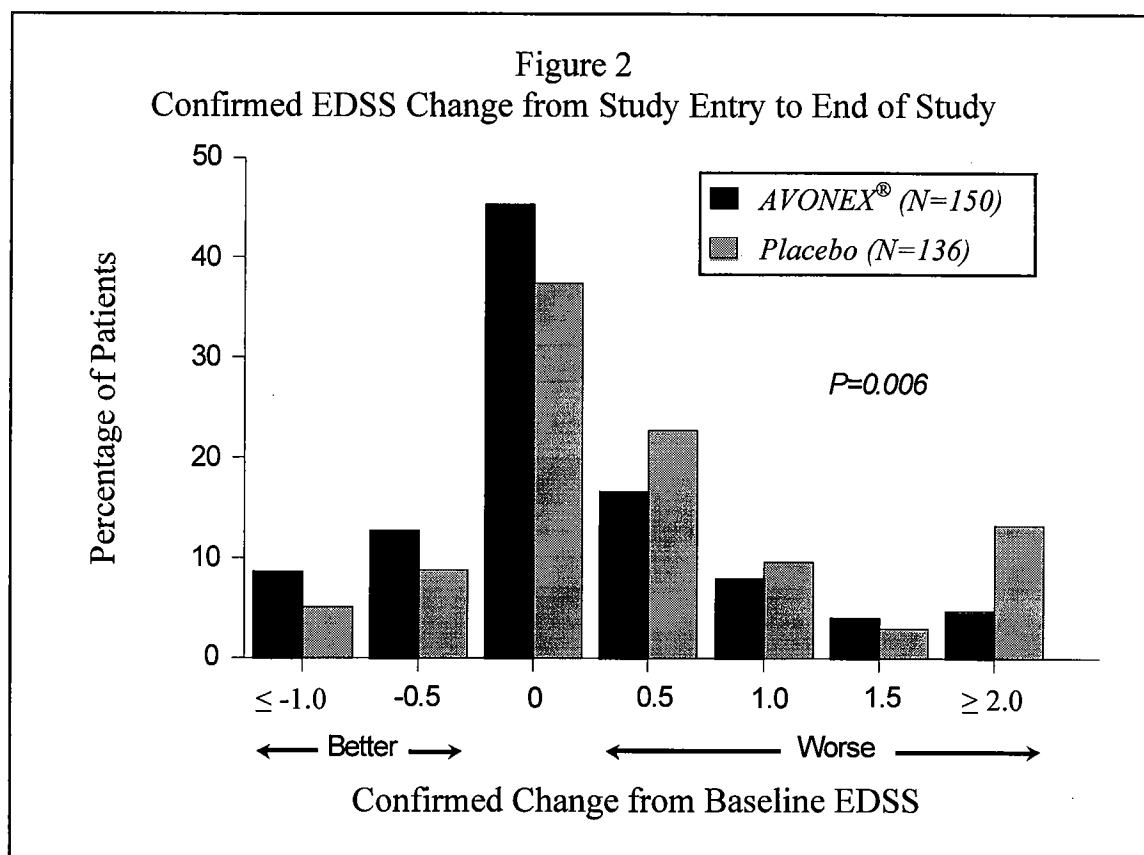
Secondary outcomes included exacerbation frequency and results of magnetic resonance imaging (MRI) scans including gadolinium (Gd)-enhanced lesion number and volume and T2-weighted (proton density) lesion volume. Additional secondary endpoints included 2 upper limb (tested in both arms) and 3 lower limb function tests.

Twenty-three of the 301 patients (8%) discontinued treatment prematurely. Of these, 1 patient treated with placebo (1%) and 6 patients treated with AVONEX® (4%) discontinued treatment due to adverse events. Thirteen of these 23 patients remained on study and were evaluated for clinical endpoints.



*Note: Disability progression represents at least a 1.0 point increase in EDSS score sustained for at least 6 months.*

Time to onset of sustained progression in disability was significantly longer in patients treated with AVONEX® than in patients receiving placebo ( $p = 0.02$ ). The Kaplan-Meier plots of these data are presented in Figure 1. The Kaplan-Meier estimate of the percentage of patients progressing by the end of 2 years was 34.9% for placebo-treated patients and 21.9% for AVONEX®-treated patients, indicating a slowing of the disease process. This represents a 37% relative reduction in the risk of accumulating disability in the AVONEX®-treated group compared to the placebo-treated group.



The distribution of confirmed EDSS change from study entry (baseline) to the end of the study is shown in Figure 2. There was a statistically significant difference between treatment groups in confirmed change for patients with at least 2 scheduled visits (136 placebo-treated and 150 AVONEX®-treated patients;  $p = 0.006$ ; see Table 1).

The rate and frequency of exacerbations were determined as secondary outcomes. For all patients included in the study, irrespective of time on study, the annual exacerbation rate was 0.67 per year in the AVONEX®-treated group and 0.82 per year in the placebo-treated group ( $p = 0.04$ ).

AVONEX® treatment significantly decreased the frequency of exacerbations in the subset of patients who were enrolled in the study for at least 2 years (87 placebo-treated patients and 85 AVONEX®-treated patients;  $p = 0.03$ ; see Table 1).

Gd-enhanced and T2-weighted (proton density) MRI scans of the brain were obtained in most patients at baseline and at the end of 1 and 2 years of treatment. Gd-enhancing

lesions seen on brain MRI scans represent areas of breakdown of the blood brain barrier thought to be secondary to inflammation. Patients treated with AVONEX® demonstrated significantly lower Gd-enhanced lesion number after 1 and 2 years of treatment ( $p \leq 0.05$ ; see Table 1). The volume of Gd-enhanced lesions was also analyzed, and showed similar treatment effects ( $p \leq 0.03$ ). Percentage change in T2-weighted lesion volume from study entry to Year 1 was significantly lower in AVONEX®-treated than placebo-treated patients ( $p = 0.02$ ). A significant difference in T2-weighted lesion volume change was not seen between study entry and Year 2.

The exact relationship between MRI findings and the clinical status of patients is unknown. The prognostic significance of MRI findings in these studies has not been evaluated.

Of the limb function tests, only 1 demonstrated a statistically significant difference between treatment groups (favoring AVONEX®). A summary of the effects of AVONEX® on the clinical and MRI endpoints of this study is presented in Table 1.

Table 1  
Clinical and MRI Endpoints in Study 1

Endpoint	Placebo	AVONEX®	P-Value
<u>PRIMARY ENDPOINT:</u>			
Time to sustained progression in disability (N: 143, 158) <sup>1</sup>	--- See Figure 1 ---		0.02 <sup>2</sup>
Percentage of patients progressing in disability at 2 years (Kaplan-Meier estimate) <sup>1</sup>	34.9%	21.9%	
<u>SECONDARY ENDPOINTS:</u>			
<u>DISABILITY</u>			
Mean confirmed change in EDSS from study entry to end of study (N: 136, 150) <sup>1</sup>	0.50	0.20	0.006 <sup>3</sup>
<u>EXACERBATIONS</u>			
Number of exacerbations in subset completing 2 years (N: 87, 85)			
0	26%	38%	0.03 <sup>3</sup>
1	30%	31%	
2	11%	18%	
3	14%	7%	
≥ 4	18%	7%	
Percentage of patients exacerbation-free in subset completing 2 years (N: 87, 85)	26%	38%	0.10 <sup>4</sup>
Annual exacerbation rate (N: 143, 158) <sup>1</sup>	0.82	0.67	0.04 <sup>5</sup>

Table 1 (continued)  
Clinical and MRI Endpoints in Study 1

Endpoint	Placebo	AVONEX®	P-Value
<u>MRI</u>			
Number of Gd-enhanced lesions:			
At study entry (N: 132, 141)			
Mean (Median)	2.3 (1.0)	3.2 (1.0)	
Range	0-23	0-56	
Year 1 (N: 123, 134)			
Mean (Median)	1.6 (0)	1.0 (0)	0.02 <sup>3</sup>
Range	0-22	0-28	
Year 2 (N: 82, 83)			
Mean (Median)	1.6 (0)	0.8 (0)	0.05 <sup>3</sup>
Range	0-34	0-13	
T2 lesion volume:			
Percentage change from study entry to Year 1 (N: 116, 123)			
Median	-3.3%	-13.1%	0.02 <sup>3</sup>
Percentage change from study entry to Year 2 (N: 83, 81)			
Median	-6.5%	-13.2%	0.36 <sup>3</sup>

Note: (N: , ) denotes the number of evaluable placebo and AVONEX® patients, respectively.

<sup>1</sup>Patient data included in this analysis represent variable periods of time on study.

<sup>2</sup>Analyzed by Mantel-Cox (logrank) test.

<sup>3</sup>Analyzed by Mann-Whitney rank-sum test.

<sup>4</sup>Analyzed by Cochran-Mantel-Haenszel test.

<sup>5</sup>Analyzed by likelihood ratio test.

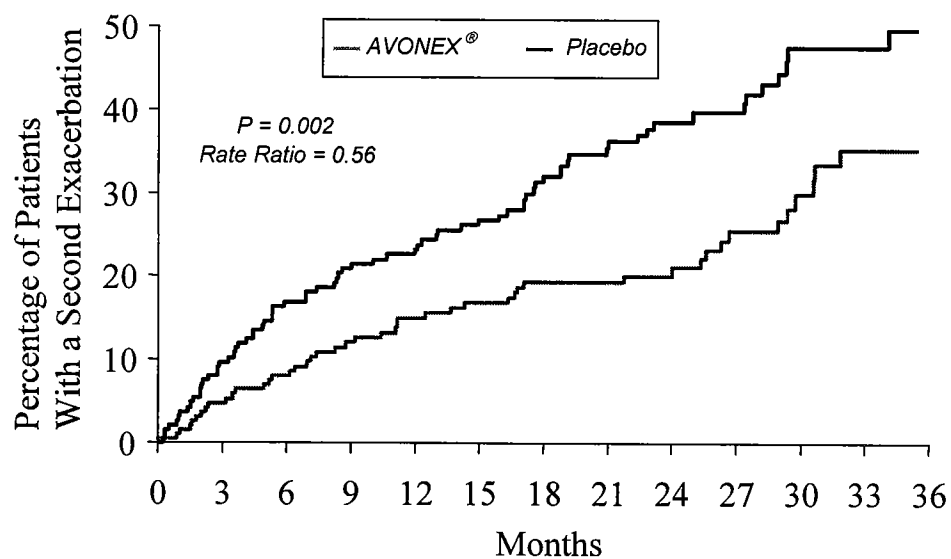
In Study 2, 383 patients who had recently experienced an isolated demyelinating event involving the optic nerve, spinal cord, or brainstem/cerebellum, and who had lesions typical of multiple sclerosis on brain MRI, received either 30 mcg AVONEX® (n = 193) or placebo (n = 190) by IM injection once weekly. All patients received intravenous steroid treatment for the initiating clinical exacerbation. Patients were enrolled into the study over a two-year period and followed for up to three years or until they developed a second clinical exacerbation in an anatomically distinct region of the central nervous system. Sixteen percent of subjects on AVONEX® and 14% of subjects on placebo withdrew from the study for a reason other than the development of a second exacerbation<sup>2</sup>.

The primary outcome measure was time to development of a second exacerbation in an anatomically distinct region of the central nervous system. Secondary outcomes were brain MRI measures, including the cumulative increase in the number of new or enlarging T2 lesions, T2 lesion volume compared to baseline at 18 months, and the number of Gd-enhancing lesions at 6 months.

Time to development of a second exacerbation was significantly delayed in patients treated with AVONEX® compared to placebo (p = 0.002). The Kaplan-Meier estimates of the percentage of patients developing an exacerbation within 24 months were 38.6% in the placebo group and 21.1% in the AVONEX® group (Figure 3). The relative rate of developing a second exacerbation in the AVONEX® group was 0.56 of the rate in the placebo group (95% confidence interval 0.38 to 0.81). The brain MRI findings are described in Table 2.

Figure 3

Onset of Second Exacerbation by Time on Study  
(Kaplan-Meier Methodology)



**Number of Subjects at Risk**

AVONEX® group	193	164	143	112	73	41
Placebo group	190	146	131	98	58	26



Table 2  
Brain MRI Data According to Treatment Group

	AVONEX <sup>®</sup>	Placebo
<u>CHANGE IN T2 VOLUME @18 MONTHS:</u>	N = 119	N = 109
Actual Change (mm <sup>3</sup> ) <sup>1*</sup>		
Median (25 <sup>th</sup> %, 75 <sup>th</sup> %)	28 (-576, 397)	313 (5, 1140)
Percentage Change <sup>1*</sup>		
Median (25 <sup>th</sup> %, 75 <sup>th</sup> %)	1 (-24, 29)	16 (0, 53)
<u>NUMBER OF NEW OR ENLARGING T2 LESIONS @ 18 MONTHS<sup>1*</sup>:</u>	N = 132	N = 119
	N (%)	N (%)
0	62 (47)	22 (18)
1-3	41 (31)	47 (40)
≥4	29 (22)	50 (42)
Mean (SD)	2.13 (3.19)	4.97 (7.71)
<u>NUMBER OF GD-ENHANCING LESIONS @ 6 MONTHS<sup>2*</sup>:</u>	N = 165	N = 152
	N (%)	N (%)
0	115 (70)	93 (61)
1	27 (16)	16 (11)
>1	23 (14)	43 (28)
Mean (SD)	0.87 (2.28)	1.49 (3.14)

<sup>1</sup> P value <0.001

<sup>2</sup> P value <0.03

\* P value from a Mann-Whitney rank-sum test

## INDICATIONS AND USAGE

AVONEX<sup>®</sup> (Interferon beta-1a) is indicated for the treatment of patients with relapsing forms of multiple sclerosis to slow the accumulation of physical disability and decrease the frequency of clinical exacerbations. Patients with multiple sclerosis in whom efficacy has been demonstrated include patients who have experienced a first clinical episode and have MRI features consistent with multiple sclerosis. Safety and efficacy in patients with chronic progressive multiple sclerosis have not been established.

## **CONTRAINDICATIONS**

AVONEX® is contraindicated in patients with a history of hypersensitivity to natural or recombinant interferon beta, or any other component of the formulation.

The lyophilized vial formulation of AVONEX® is contraindicated in patients with a history of hypersensitivity to albumin (human).

## **WARNINGS**

### **Depression and Suicide**

AVONEX® should be used with caution in patients with depression or other mood disorders, conditions that are common with multiple sclerosis. Depression and suicide have been reported to occur with increased frequency in patients receiving interferon compounds, including AVONEX®. Patients treated with AVONEX® should be advised to report immediately any symptoms of depression and/or suicidal ideation to their prescribing physicians. If a patient develops depression or other severe psychiatric symptoms, cessation of AVONEX® therapy should be considered. In Study 2, AVONEX®-treated patients were more likely to experience depression than placebo-treated patients. An equal incidence of depression was seen in the placebo-treated and AVONEX®-treated patients in Study 1. Additionally, there have been post-marketing reports of depression, suicidal ideation and/or development of new or worsening of pre-existing other psychiatric disorders, including psychosis. Some of these patients improved upon cessation of AVONEX® dosing.

### **Anaphylaxis**

Anaphylaxis has been reported as a rare complication of AVONEX® use. Other allergic reactions have included dyspnea, orolingual edema, skin rash and urticaria (see ADVERSE REACTIONS).

### **Decreased Peripheral Blood Counts**

Decreased peripheral blood counts in all cell lines, including rare pancytopenia and thrombocytopenia, have been reported from post-marketing experience (see ADVERSE REACTIONS). Some cases of thrombocytopenia have had nadirs below 10,000/ $\mu$ L. Some

cases reoccur with rechallenge (see ADVERSE REACTIONS). Patients should be monitored for signs of these disorders (see Precautions: Laboratory Tests).

### **Hepatic Injury**

Severe hepatic injury, including cases of hepatic failure, has been reported rarely in patients taking AVONEX<sup>®</sup>. Asymptomatic elevation of hepatic transaminases has also been reported, and in some patients has recurred upon rechallenge with AVONEX<sup>®</sup>. In some cases, these events have occurred in the presence of other drugs that have been associated with hepatic injury. The potential risk of AVONEX<sup>®</sup> used in combination with known hepatotoxic drugs or other products (e.g. alcohol) should be considered prior to AVONEX<sup>®</sup> administration, or when adding new agents to the regimen of patients already on AVONEX<sup>®</sup>. Patients should be monitored for signs of hepatic injury (see Precautions: Laboratory Tests).

### **Albumin (Human)**

The lyophilized vial of AVONEX<sup>®</sup> contains albumin, a derivative of human blood. Based on effective donor screening and product manufacturing processes, it carries an extremely remote risk for transmission of viral diseases. A theoretical risk for transmission of Creutzfeldt-Jakob disease (CJD) also is considered extremely remote. No cases of transmission of viral diseases or CJD have been identified for albumin. The prefilled syringe of AVONEX<sup>®</sup> does not contain albumin.

## **PRECAUTIONS**

### **Seizures**

Caution should be exercised when administering AVONEX<sup>®</sup> to patients with pre-existing seizure disorders. In the two placebo-controlled studies in multiple sclerosis, 4 patients receiving AVONEX<sup>®</sup> experienced seizures, while no seizures occurred in the placebo group. Three of these 4 patients had no prior history of seizure (see ADVERSE REACTIONS). It is not known whether these events were related to the effects of multiple sclerosis alone, to AVONEX<sup>®</sup>, or to a combination of both. The effect of AVONEX<sup>®</sup> administration on the medical management of patients with seizure disorder is unknown.

### **Cardiomyopathy and Congestive Heart Failure**

Patients with cardiac disease, such as angina, congestive heart failure, or arrhythmia, should be closely monitored for worsening of their clinical condition during initiation and continued treatment with AVONEX®. While AVONEX® does not have any known direct-acting cardiac toxicity, during the post-marketing period infrequent cases of congestive heart failure, cardiomyopathy, and cardiomyopathy with congestive heart failure have been reported in patients without known predisposition to these events, and without other known etiologies being established. In rare cases, these events have been temporally related to the administration of AVONEX®. In some of these instances recurrence upon rechallenge was observed.

### **Autoimmune Disorders**

Autoimmune disorders of multiple target organs have been reported post-marketing including idiopathic thrombocytopenia, hyper- and hypothyroidism, and rare cases of autoimmune hepatitis have also been reported. Patients should be monitored for signs of these disorders (see Precautions: Laboratory Tests) and appropriate treatment implemented when observed.

### **Information to Patients**

All patients should be instructed to read the AVONEX® Medication Guide supplied to them. Patients should be cautioned not to change the dosage or the schedule of administration without medical consultation.

Patients should be informed of the most serious (see WARNINGS) and the most common adverse events associated with AVONEX® administration, including symptoms associated with flu syndrome (see ADVERSE REACTIONS). Symptoms of flu syndrome are most prominent at the initiation of therapy and decrease in frequency with continued treatment. Concurrent use of analgesics and/or antipyretics may help ameliorate flu-like symptoms on treatment days.

Patients should be cautioned to report depression or suicidal ideation (see WARNINGS).

Patients should be advised about the abortifacient potential of AVONEX® (see Precautions: Pregnancy - Teratogenic Effects). If a woman becomes pregnant while taking AVONEX®, she should be advised to consider enrolling in the AVONEX® Pregnancy Registry by calling 1-800-456-2255.

When a physician determines that AVONEX® can be used outside of the physician's office, persons who will be administering AVONEX® should receive instruction in reconstitution and injection, including the review of the injection procedures. If a patient is to self-administer, the physical ability of that patient to self-inject intramuscularly should be assessed. The first injection should be performed under the supervision of a qualified health care professional. A puncture-resistant container for disposal of needles and syringes should be used. Patients should be instructed in the technique and importance of proper syringe and needle disposal and be cautioned against reuse of these items.

### **Laboratory Tests**

In addition to those laboratory tests normally required for monitoring patients with multiple sclerosis, complete blood and differential white blood cell counts, platelet counts, and blood chemistries, including liver function tests, are recommended during AVONEX® therapy (see WARNINGS: Decreased Peripheral Blood Counts and PRECAUTIONS: Cardiomyopathy and Congestive Heart Failure, and Autoimmune Disorders). During the placebo-controlled studies in multiple sclerosis, these tests were performed at least every 6 months. There were no significant differences between the placebo and AVONEX® groups in the incidence of liver enzyme elevation, leukopenia, or thrombocytopenia. However, these are known to be dose-related laboratory abnormalities associated with the use of interferons. Patients with myelosuppression may require more intensive monitoring of complete blood cell counts, with differential and platelet counts. Thyroid function should be monitored periodically. If patients have or develop symptoms of thyroid dysfunction (hypo- or hyperthyroidism), thyroid function tests should be performed according to standard medical practice.

## **Drug Interactions**

No formal drug interaction studies have been conducted with AVONEX®. In the placebo-controlled studies in multiple sclerosis, corticosteroids or ACTH were administered for treatment of exacerbations in some patients concurrently receiving AVONEX®. In addition, some patients receiving AVONEX® were also treated with anti-depressant therapy and/or oral contraceptive therapy. No unexpected adverse events were associated with these concomitant therapies. However, the potential for hepatic injury should be considered when AVONEX® is used in combination with other products associated with hepatic injury, or when new agents are added to the regimen of patients already on AVONEX® (see WARNINGS: Hepatic Injury).

## **Carcinogenesis, Mutagenesis, and Impairment of Fertility**

*Carcinogenesis:* No carcinogenicity data for AVONEX® are available in animals or humans.

*Mutagenesis:* AVONEX® was not mutagenic when tested in the Ames bacterial test and in an *in vitro* cytogenetic assay in human lymphocytes in the presence and absence of metabolic activation. These assays are designed to detect agents that interact directly with and cause damage to cellular DNA. AVONEX® is a glycosylated protein that does not directly bind to DNA.

*Impairment of Fertility:* No studies were conducted to evaluate the effects of AVONEX® on fertility in normal women or women with multiple sclerosis. It is not known whether AVONEX® can affect human reproductive capacity.

Menstrual irregularities were observed in monkeys administered AVONEX® at a dose 100 times the recommended weekly human dose (based upon a body surface area comparison). Anovulation and decreased serum progesterone levels were also noted transiently in some animals. These effects were reversible after discontinuation of drug.

Treatment of monkeys with AVONEX® at 2 times the recommended weekly human dose (based upon a body surface area comparison) had no effects on cycle duration or ovulation.

The accuracy of extrapolating animal doses to human doses is not known. In the placebo-controlled studies in multiple sclerosis, 5% of patients receiving placebo and 6% of patients receiving AVONEX® experienced menstrual disorder. If menstrual irregularities occur in humans, it is not known how long they will persist following treatment.

### **Pregnancy - Teratogenic Effects**

*Pregnancy Category C:* The reproductive toxicity of AVONEX® has not been studied in animals or humans. In pregnant monkeys given AVONEX® at 100 times the recommended weekly human dose (based upon a body surface area comparison), no teratogenic or other adverse effects on fetal development were observed. Abortifacient activity was evident following 3 to 5 doses at this level. No abortifacient effects were observed in monkeys treated at 2 times the recommended weekly human dose (based upon a body surface area comparison). Although no teratogenic effects were seen in these studies, it is not known if teratogenic effects would be observed in humans. There are no adequate and well-controlled studies with interferons in pregnant women. If a woman becomes pregnant or plans to become pregnant while taking AVONEX®, she should be informed of the potential hazards to the fetus, and discontinuation of AVONEX® therapy should be considered.

If a woman becomes pregnant while taking AVONEX®, consider enrolling her in the AVONEX® Pregnancy Registry by calling 1-800-456-2255.

### **Nursing Mothers**

It is not known whether AVONEX® is excreted in human milk. Because of the potential of serious adverse reactions in nursing infants, a decision should be made to either discontinue nursing or to discontinue AVONEX®.

### **Pediatric Use**

Safety and effectiveness of AVONEX® in pediatric patients below the age of 18 years have not been evaluated.

### **Geriatric Use**

Clinical studies of AVONEX® did not include sufficient numbers of patients aged 65 and over to determine whether they respond differently than younger patients.

## ADVERSE REACTIONS

Depression, suicidal ideation, and new or worsening other psychiatric disorders have been observed to be increased in patients using interferon compounds including AVONEX® (see WARNINGS: Depression and Suicide). Anaphylaxis and other allergic reactions have been reported in patients using AVONEX® (see WARNINGS: Anaphylaxis). Decreased peripheral blood counts have been reported in patients using AVONEX® (see WARNINGS: Decreased Peripheral Blood Counts). Hepatic injury, including hepatic failure, hepatitis, and elevated serum hepatic enzyme levels, has been reported in post-marketing experience (see WARNINGS: Hepatic Injury). Seizures, cardiovascular adverse events, and autoimmune disorders also have been reported in association with the use of AVONEX® (see Precautions).

The adverse reactions most commonly reported in patients associated with the use of AVONEX® were flu-like and other symptoms occurring within hours to days following an injection. Symptoms can include myalgia, fever, fatigue, headaches, chills, nausea, and vomiting. Some patients have experienced paresthesias, hypertonia and myasthenia.

The most frequently reported adverse reactions resulting in clinical intervention (e.g., discontinuation of AVONEX®, or the need for concomitant medication to treat an adverse reaction symptom) were flu-like symptoms and depression.

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of AVONEX® cannot be directly compared to rates in clinical trials of other drugs and may not reflect the rates observed in practice.

The data described below reflect exposure to AVONEX® in 351 patients, including 319 patients exposed for 6 months, and 288 patients exposed for greater than one year in placebo-controlled trials. The mean age of patients receiving AVONEX® was 35 years, 74% were women and 89% were Caucasian. Patients received either 30 mcg AVONEX® or placebo.

Table 3 enumerates adverse events and selected laboratory abnormalities that occurred at an incidence of at least 2% higher frequency in AVONEX®-treated subjects than was



observed in the placebo group. Reported adverse events have been classified using standard COSTART terms.

**Table 3**  
**Adverse Events and Selected Laboratory Abnormalities in the Placebo-Controlled Studies**

Adverse Event	Placebo (N = 333)	AVONEX® (N = 351)
<b>Body as a Whole</b>		
Headache	55%	58%
Flu-like symptoms (otherwise unspecified)	29%	49%
Pain	21%	23%
Asthenia	18%	24%
Fever	9%	20%
Chills	5%	19%
Abdominal pain	6%	8%
Injection site pain	6%	8%
Infection	4%	7%
Injection site inflammation	2%	6%
Chest pain	2%	5%
Injection site reaction	1%	3%
Toothache	1%	3%
<b>Nervous System</b>		
Depression	14%	18%
Dizziness	12%	14%
<b>Respiratory System</b>		
Upper respiratory tract infection	12%	14%
Sinusitis	12%	14%
Bronchitis	5%	8%
<b>Digestive System</b>		
Nausea	19%	23%
<b>Musculoskeletal System</b>		
Myalgia	22%	29%
Arthralgia	6%	9%
<b>Urogenital</b>		
Urinary tract infection	15%	17%
Urine constituents abnormal	0%	3%
<b>Skin and Appendages</b>		
Alopecia	2%	4%
<b>Special Senses</b>		
Eye disorder	2%	4%
<b>Hemic and Lymphatic System</b>		
Injection site ecchymosis	4%	6%
Anemia	1%	4%
<b>Cardiovascular System</b>		
Migraine	3%	5%
Vasodilation	0%	2%

No AVONEX<sup>®</sup>-treated patients attempted suicide in the two placebo-controlled studies. In Study 2, AVONEX<sup>®</sup>-treated patients were more likely to experience depression than placebo-treated patients (20% in AVONEX<sup>®</sup> group vs. 13% in placebo group). The incidences of depression in the placebo-treated and AVONEX<sup>®</sup>-treated patients in Study 1 were similar. In Study 1, suicidal tendency was seen more frequently in AVONEX<sup>®</sup>-treated patients (4% in AVONEX<sup>®</sup> group vs. 1% in placebo group) (see WARNINGS).

### **Seizures**

Seizures have been reported in 4 of 351 AVONEX<sup>®</sup>-treated patients in the placebo-controlled studies, compared to none in the placebo-treated patients (see Precautions: Seizures).

### **Post-Marketing Experience**

The following adverse events have been identified and reported during post-approval use of AVONEX<sup>®</sup>: New or worsening other psychiatric disorders, and anaphylaxis (see WARNINGS). Autoimmune disorders including autoimmune hepatitis, idiopathic thrombocytopenia, hyper- and hypothyroidism, and seizures in patients without prior history (see Precautions).

Infrequent reports of congestive heart failure, cardiomyopathy, and cardiomyopathy with congestive heart failure with rare cases being temporally related to the administration of AVONEX<sup>®</sup> (see Precautions: Cardiomyopathy and Congestive Heart Failure).

Decreased peripheral blood counts in all cell lines, including rare pancytopenia and thrombocytopenia (see WARNINGS: Decreased Peripheral Blood Counts). Some cases of thrombocytopenia have had nadirs below 10,000/ $\mu$ L. Some of these cases reoccur upon rechallenge.

Hepatic injury, including hepatic failure and elevated serum hepatic enzyme levels, some of which have been severe, has been reported post-marketing (see WARNINGS: Hepatic Injury).

Meno- and metrorrhagia, rash (including vesicular rash), and rare cases of injection site abscess or cellulitis that may require surgical intervention have also been reported in post-marketing experience.

Because reports of these reactions are voluntary and the population is of an uncertain size, it is not always possible to reliably estimate the frequency of the event or establish a causal relationship to drug exposure.

### **Adverse Reactions Associated with Subcutaneous Use**

AVONEX® has also been evaluated in 290 patients with diseases other than multiple sclerosis, primarily chronic viral hepatitis B and C, in which the doses studied ranged from 15 mcg to 75 mcg, given SC, 3 times a week, for up to 6 months. Inflammation at the site of the subcutaneous injection was observed in 52% of treated patients in these studies. Subcutaneous injections were also associated with the following local reactions: injection site necrosis, injection site atrophy, injection site edema and injection site hemorrhage. None of the above was observed in the multiple sclerosis patients participating in Study 1. Injection site edema and injection site hemorrhage were observed in multiple sclerosis patients participating in Study 2.

### **Immunogenicity**

As with all therapeutic proteins, there is a potential for immunogenicity. In recent studies assessing immunogenicity in multiple sclerosis patients administered AVONEX® for at least 1 year, 5% (21 of 390 patients) showed the presence of neutralizing antibodies at one or more times. The clinical significance of neutralizing antibodies to AVONEX® is unknown.

These data reflect the percentage of patients whose test results were considered positive for antibodies to AVONEX® using a two-tiered assay (ELISA binding assay followed by an antiviral cytopathic effect assay), and are highly dependent on the sensitivity and specificity of the assay. Additionally, the observed incidence of neutralizing activity in an assay may be influenced by several factors including sample handling, timing of sample collection, concomitant medications, and underlying disease. For these reasons, comparison of the incidence of antibodies to AVONEX® with the incidence of antibodies to other products may be misleading.

Anaphylaxis has been reported as a rare complication of AVONEX<sup>®</sup> use. Other allergic reactions have included dyspnea, orolingual edema, skin rash and urticaria (see WARNINGS: Anaphylaxis).

#### **DRUG ABUSE AND DEPENDENCE**

There is no evidence that abuse or dependence occurs with AVONEX<sup>®</sup> therapy. However, the risk of dependence has not been systematically evaluated.

#### **OVERDOSAGE**

Safety of doses higher than 60 mcg once a week have not been adequately evaluated. The maximum amount of AVONEX<sup>®</sup> that can be safely administered has not been determined.

#### **DOSAGE AND ADMINISTRATION**

The recommended dosage of AVONEX<sup>®</sup> (Interferon beta-1a) is 30 mcg injected intramuscularly once a week.

AVONEX<sup>®</sup> is intended for use under the guidance and supervision of a physician. Patients may self-inject only if their physician determines that it is appropriate and with medical follow-up, as necessary, after proper training in intramuscular injection technique. Sites for injection include the thigh or upper arm (see Medication Guide).

A 25 gauge, 1" needle for intramuscular injection may be substituted for the 23 gauge, 1 ¼" needle by the prescribing physician, if deemed appropriate.

#### **Reconstitution of AVONEX<sup>®</sup> Vials**

Use appropriate aseptic technique during the preparation of AVONEX®. To reconstitute lyophilized AVONEX®, use a sterile syringe and MICRO PIN® to inject 1.1 mL of the supplied diluent, Sterile Water for Injection, USP, into the AVONEX® vial. Gently swirl the vial of AVONEX® to dissolve the drug completely. **DO NOT SHAKE.** The reconstituted solution should be clear to slightly yellow without particles. Inspect the reconstituted product visually prior to use. Discard the product if it contains particulate matter or is discolored. Each vial of reconstituted solution contains 30 mcg/1.0 mL Interferon beta-1a.

Withdraw 1.0 mL of reconstituted solution from the vial into a sterile syringe. Replace the cover on the MICRO PIN® attach the sterile needle and inject the solution intramuscularly. The AVONEX® and diluent vials are for single-use only; unused portions should be discarded.

#### Using Avonex® Prefilled Syringes

The AVONEX® prefilled syringe should be held upright (cap faces up). Remove the cap by bending it at a 90° angle until it snaps free. Attach the needle and inject the solution intramuscularly. The AVONEX® prefilled syringe is for single-use only.

### **HOW SUPPLIED**

#### 30 mcg Lyophilized Powder Vial

A vial of AVONEX® is supplied as a lyophilized powder in a single-use vial containing 33 mcg (6.6 million IU) of Interferon beta-1a; 16.5 mg Albumin (Human), USP; 6.4 mg Sodium Chloride, USP; 6.3 mg Dibasic Sodium Phosphate, USP; and 1.3 mg Monobasic Sodium Phosphate, USP, and is preservative-free. Diluent is supplied in a single-use vial (Sterile Water for Injection, USP).

AVONEX® lyophilized vials are available in the following package configuration (NDC 59627-001-03): A package containing four Administration Dose Packs (each containing one vial of AVONEX®, one 10 mL diluent vial, two alcohol wipes, one gauze pad, one 3 mL syringe, one MICRO PIN®\* vial access pin, one 23 gauge, 1¼ inch needle, and one adhesive bandage).

#### 30 mcg Prefilled Syringe

A prefilled syringe of AVONEX<sup>®</sup> is supplied as a sterile liquid albumin-free formulation containing 30 mcg of Interferon beta-1a, 0.79 mg Sodium Acetate Trihydrate, USP; 0.25 mg Glacial Acetic Acid, USP; 15.8 mg Arginine Hydrochloride, USP; and 0.025 mg Polysorbate 20 in Water for Injection, USP. Each prefilled glass syringe contains 0.5 mL for IM injection.

AVONEX<sup>®</sup> prefilled syringes are available in the following package configuration (NDC 59627-002-05): A package containing four Administration Dose Packs (each containing one single-use syringe of AVONEX<sup>®</sup> and one 23 gauge, 1¼ inch needle), and a recloseable accessory pouch containing 4 alcohol wipes, 4 gauze pads, and 4 adhesive bandages.

### **Stability and Storage**

#### **30 mcg Lyophilized Powder Vial**

Vials of AVONEX<sup>®</sup> should be stored in a 2-8°C (36-46°F) refrigerator. Should refrigeration be unavailable, vials of AVONEX<sup>®</sup> can be stored at 25°C (77°F) for a period of up to 30 days. **DO NOT EXPOSE TO HIGH TEMPERATURES. DO NOT FREEZE.** Protect from light. Do not use beyond the expiration date stamped on the vial. Following reconstitution, it is recommended the product be used as soon as possible within 6 hours stored at 2-8°C (36-46°F). **DO NOT FREEZE RECONSTITUTED AVONEX<sup>®</sup>.**

#### **30 mcg Prefilled Syringe**

AVONEX<sup>®</sup> in prefilled syringes should be stored in a 2-8°C (36-46°F) refrigerator. Once removed from the refrigerator, AVONEX<sup>®</sup> in a prefilled syringe should be allowed to warm to room temperature (about 30 minutes). Do not use external heat sources such as hot water to warm AVONEX<sup>®</sup> in a prefilled syringe. Should refrigeration be unavailable, AVONEX<sup>®</sup> in a prefilled syringe can be stored at ≤25°C (77°F) for a period up to 7 days. Once the product is removed from the refrigerator, it must not be stored above 25°C (77°F). If the product has been exposed to conditions other than those recommended, **DISCARD THE PRODUCT and DO NOT USE.** **DO NOT EXPOSE TO HIGH TEMPERATURES. DO NOT FREEZE.** Protect from light. Do not use beyond the expiration date stamped on the syringe.

### **REFERENCES**

1. Jacobs LD, et al. Intramuscular interferon beta-1a for disease progression in relapsing multiple sclerosis. Ann Neurol 1996;39(3):285-294.
2. Jacobs LD, et al. Intramuscular interferon beta-1a initiated during a first demyelinating event in multiple sclerosis. NEJM 2000;343:898-904.
3. Kurtzke JF. Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). Neurology 1983;33:1444-1452.

**AVONEX® (Interferon beta-1a)**

Manufactured by:  
BIOGEN IDEC INC.  
14 Cambridge Center  
Cambridge, MA 02142 USA  
©2006 Biogen Idec Inc. All rights reserved.  
1-800-456-2255

U.S. Patent Pending  
I61023-4 (Issue Date 10/2008)

Rx only

*\*Micro Pin® is the trademark of B. Braun Medical Inc.*



**HIGHLIGHTS OF PRESCRIBING INFORMATION**

These highlights do not include all the information needed to use Campath safely and effectively. See full prescribing information for Campath.

Campath® (alemtuzumab)  
Injection for intravenous use  
Initial U.S. Approval: 2001

**WARNING: CYTOPENIAS, INFUSION REACTIONS, and INFECTIONS**

*See full prescribing information for complete boxed warning.*

Serious, including fatal, cytopenias, infusion reactions and infections can occur (5.1 – 5.3).

- Limit doses to 30 mg (single) and 90 mg (cumulative weekly); higher doses increase risk of pancytopenia (2.1).
- Escalate dose gradually and monitor patients during infusion. Withhold therapy for Grade 3 or 4 infusion reactions (5.2).
- Administer prophylaxis against *Pneumocystis jiroveci* pneumonia (PCP) and herpes virus infections (2.2, 5.3).

**RECENT MAJOR CHANGES**

Warnings and Precautions (5.3)

10/2008

**INDICATIONS AND USAGE**

Campath is a CD52-directed cytolytic antibody indicated as a single agent for the treatment of B-cell chronic lymphocytic leukemia (B-CLL) (1).

**DOSAGE AND ADMINISTRATION**

- Administer as an IV infusion over 2 hours (2.1).
- Escalate to recommended dose of 30 mg/day three times per week for 12 weeks (2.1).
- Premedicate with oral antihistamine and acetaminophen prior to dosing (2.2).

**DOSAGE FORMS AND STRENGTHS**

30 mg/1 mL single use vial (3).

**CONTRAINDICATIONS**

None (4).

**WARNINGS AND PRECAUTIONS****Cytopenias:**

- Obtain complete blood counts (CBC) and platelet counts at weekly intervals during therapy and CD4 counts after therapy until recovery to  $\geq 200$  cells/ $\mu$ L (5.4).
- Discontinue for autoimmune or severe hematologic adverse reactions (5.1).

**Infections:**

- Campath induces severe and prolonged lymphopenia and increases risk of infection. If a serious infection occurs, withhold treatment until infection resolves (5.3).
- Do not administer live viral vaccines to patients who have recently received Campath (5.5).

**ADVERSE REACTIONS**

Most common adverse reactions ( $\geq 10\%$ ): cytopenias, infusion reactions, cytomegalovirus (CMV) and other infections, nausea, emesis, diarrhea, and insomnia (6).

To report SUSPECTED ADVERSE REACTIONS, contact Bayer HealthCare Pharmaceuticals at 1-888-842-2937 or FDA at 1-800-FDA-1088 or [www.fda.gov/medwatch](http://www.fda.gov/medwatch)

**See 17 for PATIENT COUNSELING INFORMATION**

Revised: 10/2008

**FULL PRESCRIBING INFORMATION: CONTENTS\*****WARNING: CYTOPENIAS, INFUSION REACTIONS, and INFECTIONS****1 INDICATIONS AND USAGE****2 DOSAGE AND ADMINISTRATION**

- 2.1 Dosing Schedule and Administration
- 2.2 Recommended Concomitant Medications
- 2.3 Dose Modification
- 2.4 Preparation and Administration
- 2.5 Incompatibilities

**3 DOSAGE FORMS AND STRENGTHS****4 CONTRAINDICATIONS****5 WARNINGS AND PRECAUTIONS**

- 5.1 Cytopenias
- 5.2 Infusion Reactions
- 5.3 Immunosuppression/Infections
- 5.4 Laboratory Monitoring
- 5.5 Immunization

**6 ADVERSE REACTIONS**

- 6.1 Clinical Trials Experience
- 6.2 Immunogenicity
- 6.3 Postmarketing Experience

**7 DRUG INTERACTIONS****8 USE IN SPECIFIC POPULATIONS**

- 8.1 Pregnancy
- 8.3 Nursing Mothers
- 8.4 Pediatric Use
- 8.5 Geriatric Use

**10 OVERDOSAGE****11 DESCRIPTION****12 CLINICAL PHARMACOLOGY**

- 12.1 Mechanism of Action
- 12.3 Pharmacokinetics

**13 NONCLINICAL TOXICOLOGY**

- 13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

**14 CLINICAL STUDIES**

- 14.1 Previously Untreated B-CLL Patients
- 14.2 Previously Treated B-CLL Patients

**15 REFERENCES****16 HOW SUPPLIED/STORAGE AND HANDLING****17 PATIENT COUNSELING INFORMATION**

\*Sections or subsections omitted from the full prescribing information are not listed.

## FULL PRESCRIBING INFORMATION

### WARNING: CYTOPENIAS, INFUSION REACTIONS, and INFECTIONS

**Cytopenias:** Serious, including fatal, pancytopenia/marrow hypoplasia, autoimmune idiopathic thrombocytopenia, and autoimmune hemolytic anemia can occur in patients receiving Campath. Single doses of Campath greater than 30 mg or cumulative doses greater than 90 mg per week increase the incidence of pancytopenia [see **WARNINGS AND PRECAUTIONS (5.1)**].

**Infusion Reactions:** Campath administration can result in serious, including fatal, infusion reactions. Carefully monitor patients during infusions and withhold Campath for Grade 3 or 4 infusion reactions. Gradually escalate Campath to the recommended dose at the initiation of therapy and after interruption of therapy for 7 or more days [see **DOSAGE AND ADMINISTRATION (2)** and **WARNINGS AND PRECAUTIONS (5.2)**].

**Infections:** Serious, including fatal, bacterial, viral, fungal, and protozoan infections can occur in patients receiving Campath. Administer prophylaxis against *Pneumocystis jiroveci* pneumonia (PCP) and herpes virus infections [see **DOSAGE AND ADMINISTRATION (2.2)** and **WARNINGS AND PRECAUTIONS (5.3)**].

## 1 INDICATIONS AND USAGE

Campath is indicated as a single agent for the treatment of B-cell chronic lymphocytic leukemia (B-CLL).

## 2 DOSAGE AND ADMINISTRATION

### 2.1 Dosing Schedule and Administration

- Administer as an IV infusion over 2 hours. **Do not administer as intravenous push or bolus.**
- Recommended Dosing Regimen
  - Gradually escalate to the maximum recommended single dose of 30 mg. Escalation is required at initiation of dosing or if dosing is held  $\geq 7$  days during treatment. Escalation to 30 mg ordinarily can be accomplished in 3–7 days.
  - Escalation Strategy:
    - Administer 3 mg daily until infusion reactions are  $\leq$  grade 2 [see **ADVERSE REACTIONS (6.1)**].
    - Then administer 10 mg daily until infusion reactions are  $\leq$  grade 2.
    - Then administer 30 mg/day three times per week on alternate days (e.g., Mon-Wed-Fri). The total duration of therapy, including dose escalation, is 12 weeks.
- Single doses of greater than 30 mg or cumulative doses greater than 90 mg per week increase the incidence of pancytopenia.

### 2.2 Recommended Concomitant Medications

- Premedicate with diphenhydramine (50 mg) and acetaminophen (500–1000 mg) 30 minutes prior to first infusion and each dose escalation. Institute appropriate medical management (e.g. steroids, epinephrine, meperidine) for infusion reactions as needed [see **BOXED WARNING, WARNINGS AND PRECAUTIONS (5.2)** and **ADVERSE REACTIONS (6.1)**].
- Administer trimethoprim/sulfamethoxazole DS twice daily (BID) three times per week (or equivalent) as *Pneumocystis jiroveci* pneumonia (PCP) prophylaxis.
- Administer famciclovir 250 mg BID or equivalent as herpetic prophylaxis. Continue PCP and herpes viral prophylaxis for a minimum of 2 months after completion of Campath or until the CD4+ count is  $\geq 200$  cells/ $\mu$ L, whichever occurs later [see **BOXED WARNING** and **WARNINGS AND PRECAUTIONS (5.3)**].

### 2.3 Dose Modification

- Withhold Campath during serious infection or other serious adverse reactions until resolution.

- Discontinue Campath for autoimmune anemia or autoimmune thrombocytopenia.
- There are no dose modifications recommended for lymphopenia.

### Dose Modification for Neutropenia or Thrombocytopenia

[see **WARNINGS AND PRECAUTIONS (5.1)**]

Hematologic Values	Dose Modification*
ANC $< 250/\mu$ L and/or platelet count $\leq 25,000/\mu$ L	
For first occurrence:	Withhold Campath therapy. Resume Campath at 30 mg when ANC $\geq 500/\mu$ L and platelet count $\geq 50,000/\mu$ L.
For second occurrence:	Withhold Campath therapy. Resume Campath at 10 mg when ANC $\geq 500/\mu$ L and platelet count $\geq 50,000/\mu$ L.
For third occurrence:	Discontinue Campath therapy.
$\geq 50\%$ decrease from baseline in patients initiating therapy with a baseline ANC $\leq 250/\mu$ L and/or a baseline platelet count $\leq 25,000/\mu$ L	
For first occurrence:	Withhold Campath therapy. Resume Campath at 30 mg upon return to baseline value(s).
For second occurrence:	Withhold Campath therapy. Resume Campath at 10 mg upon return to baseline value(s).
For third occurrence:	Discontinue Campath therapy.

\*If the delay between dosing is  $\geq 7$  days, initiate therapy at Campath 3 mg and escalate to 10 mg and then to 30 mg as tolerated [see **DOSAGE AND ADMINISTRATION (2.1)**].

### 2.4 Preparation and Administration

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration. If particulate matter is present or the solution is discolored, the vial should not be used. **DO NOT SHAKE VIAL.**

Use aseptic technique during the preparation and administration of Campath. Withdraw the necessary amount of Campath from the vial into a syringe.

- To prepare the 3 mg dose, withdraw 0.1 mL into a 1 mL syringe calibrated in increments of 0.01 mL.
- To prepare the 10 mg dose, withdraw 0.33 mL into a 1 mL syringe calibrated in increments of 0.01 mL.
- To prepare the 30 mg dose, withdraw 1 mL in either a 1 mL or 3 mL syringe calibrated in 0.1 mL increments.

Inject syringe contents into 100 mL sterile 0.9% Sodium Chloride USP or 5% Dextrose in Water USP. **Gently invert the bag to mix the solution.** Discard syringe.

**The vial contains no preservatives and is intended for single use only. DISCARD VIAL including any unused portion after withdrawal of dose.**

Use within 8 hours after dilution. Store diluted Campath at room temperature (15–30°C) or refrigerated (2–8°C). Protect from light.

### 2.5 Incompatibilities

Campath is compatible with polyvinylchloride (PVC) bags and PVC or polyethylene-lined PVC administration sets. Do not add or simultaneously infuse other drug substances through the same intravenous line.

## 3 DOSAGE FORMS AND STRENGTHS

30 mg/1 mL single use vial

## 4 CONTRAINDICATIONS

None

## 5 WARNINGS AND PRECAUTIONS

### 5.1 Cytopenias

Severe, including fatal, autoimmune anemia and thrombocytopenia, and prolonged myelosuppression have been reported in patients receiving Campath.

In addition, hemolytic anemia, pure red cell aplasia, bone marrow aplasia, and hypoplasia have been reported after treatment with Campath at the recommended dose. Single doses of Campath greater than 30 mg or cumulative doses greater than 90 mg per week increase the incidence of pancytopenia.

Withhold Campath for severe cytopenias (except lymphopenia). Discontinue for autoimmune cytopenias or recurrent/persistent severe cytopenias (except lymphopenia) [see *DOSAGE AND ADMINISTRATION* (2.3)]. No data exist on the safety of Campath resumption in patients with autoimmune cytopenias or marrow aplasia [see *ADVERSE REACTIONS* (6.1)].

## 5.2 Infusion Reactions

Adverse reactions occurring during or shortly after Campath infusion include pyrexia, chills/rigors, nausea, hypotension, urticaria, dyspnea, rash, emesis, and bronchospasm. In clinical trials, the frequency of infusion reactions was highest in the first week of treatment. Monitor for the signs and symptoms listed above and withhold infusion for Grade 3 or 4 infusion reactions [see *ADVERSE REACTIONS* (6.1)].

The following serious, including fatal, infusion reactions have been identified in post-marketing reports: syncope, pulmonary infiltrates, acute respiratory distress syndrome (ARDS), respiratory arrest, cardiac arrhythmias, myocardial infarction, acute cardiac insufficiency, cardiac arrest, angioedema, and anaphylactoid shock.

Initiate Campath according to the recommended dose-escalation scheme [see *DOSAGE AND ADMINISTRATION* (2)]. Premedicate patients with an antihistamine and acetaminophen prior to dosing. Institute medical management (e.g., glucocorticoids, epinephrine, meperidine) for infusion reactions as needed [see *DOSAGE AND ADMINISTRATION* (2.2)]. If therapy is interrupted for 7 or more days, reinstitute Campath with gradual dose escalation [see *DOSAGE AND ADMINISTRATION* (2.3) and *ADVERSE REACTIONS* (6)].

## 5.3 Immunosuppression/Infections

Campath treatment results in severe and prolonged lymphopenia with a concomitant increased incidence of opportunistic infections [see *ADVERSE REACTIONS* (6.1)]. Administer PCP and herpes viral prophylaxis during Campath therapy and for a minimum of 2 months after completion of Campath or until the CD4+ count is  $\geq 200$  cells/ $\mu$ L, whichever occurs later [see *DOSAGE AND ADMINISTRATION* (2.2)]. Prophylaxis does not eliminate these infections.

Routinely monitor patients for CMV infection during Campath treatment and for at least 2 months following completion of treatment. Withhold Campath for serious infections and during antiviral treatment for CMV infection or confirmed CMV viremia (defined as polymerase chain reaction (PCR) positive CMV in  $\geq 2$  consecutive samples obtained 1 week apart) [see *ADVERSE REACTIONS* (6.1)]. Initiate therapeutic ganciclovir (or equivalent) for CMV infection or confirmed CMV viremia [see *DOSAGE AND ADMINISTRATION* (2.3)].

Administer only irradiated blood products to avoid transfusion associated Graft versus Host Disease (TAGVHD), unless emergent circumstances dictate immediate transfusion.<sup>1</sup>

In patients receiving Campath as initial therapy, recovery of CD4+ counts to  $\geq 200$  cells/ $\mu$ L occurred by 6 months post-treatment; however at 2 months post-treatment, the median was 183 cells/ $\mu$ L. In previously treated patients receiving Campath, the median time to recovery of CD4+ counts to  $\geq 200$  cells/ $\mu$ L was 2 months; however, full recovery (to baseline) of CD4+ and CD8+ counts may take more than 12 months [see *BOXED WARNING* and *ADVERSE REACTIONS* (6)].

## 5.4 Laboratory Monitoring

Obtain complete blood counts (CBC) at weekly intervals during Campath therapy and more frequently if worsening anemia, neutropenia, or thrombocytopenia occurs. Assess CD4+ counts after treatment until recovery to  $\geq 200$  cells/ $\mu$ L [see *WARNINGS AND PRECAUTIONS* (5.3) and *ADVERSE REACTIONS* (6)].

## 5.5 Immunization

The safety of immunization with live viral vaccines following Campath therapy has not been studied. Do not administer live viral vaccines to patients who have recently received Campath. The ability to generate an immune response to any vaccine following Campath therapy has not been studied.

## 6 ADVERSE REACTIONS

The following adverse reactions are discussed in greater detail in other sections of the label:

- Cytopenias [see *WARNINGS AND PRECAUTIONS* (5.1)]
- Infusion Reactions [see *WARNINGS AND PRECAUTIONS* (5.2)]
- Immunosuppression/Infections [see *WARNINGS AND PRECAUTIONS* (5.3)]

The most common adverse reactions with Campath are: infusion reactions (pyrexia, chills, hypotension, urticaria, nausea, rash, tachycardia, dyspnea), cytopenias (neutropenia, lymphopenia, thrombocytopenia, anemia), infections (CMV viremia, CMV infection, other infections), gastrointestinal symptoms (nausea, emesis, abdominal pain), and neurological symptoms (insomnia, anxiety). The most common serious adverse reactions are cytopenias, infusion reactions, and immunosuppression/infections.

### 6.1 Clinical Trials Experience

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice.

The data below reflect exposure to Campath in 296 patients with CLL of whom 147 were previously untreated and 149 received at least 2 prior chemotherapy regimens. The median duration of exposure was 11.7 weeks for previously untreated patients and 8 weeks for previously treated patients.

**Lymphopenia:** Severe lymphopenia and a rapid and sustained decrease in lymphocyte subsets occurred in previously untreated and previously treated patients following administration of Campath. In previously untreated patients, the median CD4+ was 0 cells/ $\mu$ L at one month after treatment and 238 cells/ $\mu$ L [25–75% interquartile range 115 to 418 cells/ $\mu$ L] at 6 months post-treatment [see *WARNINGS AND PRECAUTIONS* (5.3)].

**Neutropenia:** In previously untreated patients, the incidence of Grade 3 or 4 neutropenia was 42% with a median time to onset of 31 days and a median duration of 37 days. In previously treated patients, the incidence of Grade 3 or 4 neutropenia was 64% with a median duration of 28 days. Ten percent of previously untreated patients and 17% of previously treated patients received granulocyte colony stimulating factors.

**Anemia:** In previously untreated patients, the incidence of Grade 3 or 4 anemia was 12% with a median time to onset of 31 days and a median duration of 8 days. In previously treated patients, the incidence of Grade 3 or 4 anemia was 38%. Seventeen percent of previously untreated patients and 66% of previously treated patients received either erythropoiesis stimulating agents, transfusions or both.

**Thrombocytopenia:** In previously untreated patients, the incidence of Grade 3 or 4 thrombocytopenia was 14% with a median time to onset of 9 days and a median duration of 14 days. In previously treated patients, the incidence of Grade 3 or 4 thrombocytopenia was 52% with a median duration of 21 days. Autoimmune thrombocytopenia was reported in 2% of previously treated patients with one fatality.

**Infusion reactions:** Infusion reactions, which included pyrexia, chills, hypotension, urticaria, and dyspnea, were common. Grade 3 and 4 pyrexia and/or chills occurred in approximately 10% of previously untreated patients and in approximately 35% of previously treated patients. The occurrence of infusion reactions was greatest during the initial week of treatment and decreased with subsequent doses of Campath. All patients were pretreated with antipyretics and antihistamines; additionally, 43% of previously untreated patients received glucocorticoid pre-treatment.

**Infections:** In the study of previously untreated patients, patients were tested weekly for CMV using a PCR assay from initiation through completion of therapy, and every 2 weeks for the first 2 months following therapy. CMV infection occurred in 16% (23/147) of previously untreated patients; approximately one-third of these infections were serious or life threatening. In studies of previously treated patients in which routine CMV surveillance was not required, CMV infection was documented in 6% (9/149) of patients; nearly all of these infections were serious or life threatening.

Other infections were reported in approximately 50% of patients across all studies. Grade 3 – 5 sepsis ranged from 3% to 10% across studies and was higher in previously treated patients. Grade 3 – 4 febrile neutropenia ranged from 5 to 10% across studies and was higher in previously treated patients. Infection-related fatalities occurred in 2% of previously untreated patients and 16% of previously treated patients. There were 198 episodes of other infection in 109 previously untreated patients; 16% were bacterial, 7% were fungal, 4% were other viral, and in 73%, the organism was not identified.

**Cardiac:** Cardiac dysrhythmias occurred in approximately 14% of previously untreated patients. The majority were tachycardias and were temporally associated with infusion; dysrhythmias were Grade 3 or 4 in 1% of patients.

#### Previously Untreated Patients

Table 1 contains selected adverse reactions observed in 294 patients randomized (1:1) to receive Campath or chlorambucil as first line therapy for B-CLL. Campath was administered at a dose of 30 mg intravenously three times weekly for up to 12 weeks. The median duration of therapy was 11.7 weeks with a median weekly dose of 82 mg (25–75% interquartile range: 69 mg – 90 mg).

**Table 1**

Per Patient Incidence of Selected <sup>1</sup> Adverse Reactions in Treatment Naive B-CLL Patients				
	Campath (n=147)		Chlorambucil (n=147)	
	All Grades <sup>2</sup> %	Grades 3-4 %	All Grades %	Grades 3-4 %
<b>Blood and Lymphatic System Disorders</b>				
Lymphopenia	97	97	9	1
Neutropenia	77	42	51	26
Anemia	76	13	54	18
Thrombocytopenia	71	13	70	14
<b>General Disorders and Administration Site Conditions</b>				
Pyrexia	69	10	11	1
Chills	53	3	1	0
<b>Infections and Infestations</b>				
CMV viremia <sup>3</sup>	55	4	8	0
CMV infection	16	5	0	0
Other infections	74	21	65	10
<b>Skin and Subcutaneous Tissue Disorders</b>				
Urticaria	16	2	1	0
Rash	13	1	4	0
Erythema	4	0	1	0
<b>Vascular Disorders</b>				
Hypotension	16	1	0	0
Hypertension	14	5	2	1
<b>Nervous System Disorders</b>				
Headache	14	1	8	0
Tremor	3	0	1	0
<b>Respiratory, Thoracic and Mediastinal Disorders</b>				
Dyspnea	14	4	7	3

(Continued)

**Table 1**

(Continued)

Per Patient Incidence of Selected <sup>1</sup> Adverse Reactions in Treatment Naive B-CLL Patients				
	Campath (n=147)		Chlorambucil (n=147)	
	All Grades <sup>2</sup> %	Grades 3-4 %	All Grades %	Grades 3-4 %
<b>Gastrointestinal Disorders</b>				
Diarrhea	10	1	4	0
<b>Psychiatric Disorders</b>				
Insomnia	10	0	3	0
Anxiety	8	0	1	0
<b>Cardiac Disorders</b>				
Tachycardia	10	0	1	0

<sup>1</sup> Adverse reactions occurring at a higher relative frequency in the Campath arm

<sup>2</sup> NCI CTC version 2.0 for adverse reactions; NCI CTCAE version 3.0 for laboratory values

<sup>3</sup> CMV viremia (without evidence of symptoms) includes both cases of single PCR positive test results and of confirmed CMV viremia ( $\geq 2$  occasions in consecutive samples 1 week apart). For the latter, ganciclovir (or equivalent) was initiated per protocol.

#### Previously Treated Patients

Additional safety information was obtained from 3 single arm studies of 149 previously treated patients with CLL administered 30 mg Campath intravenously three times weekly for 4 to 12 weeks (median cumulative dose 673 mg [range 2 – 1106 mg]; median duration of therapy 8.0 weeks). Adverse reactions in these studies not listed in Table 1 that occurred at an incidence rate of  $> 5\%$  were fatigue, nausea, emesis, musculoskeletal pain, anorexia, dysesthesia, mucositis, and bronchospasm.

### **6.2 Immunogenicity**

As with all therapeutic proteins, there is potential for immunogenicity. Using an ELISA assay, anti-human antibodies (HAHA) were detected in 11 of 133 (8.3%) previously untreated patients. In addition, two patients were weakly positive for neutralizing activity. Limited data suggest that the anti-Campath antibodies did not adversely affect tumor response. Four of 211 (1.9%) previously-treated patients were found to have antibodies to Campath following treatment.

The incidence of antibody formation is highly dependent on the sensitivity and specificity of the assay. Additionally, the observed incidence of antibody (including neutralizing antibody) positivity in an assay may be influenced by several factors including assay methodology, sample handling, timing of sample collection, concomitant medications, and underlying disease. For these reasons, comparison of the incidence of antibodies to Campath with the incidence of antibodies to other products may be misleading.

### **6.3 Postmarketing Experience**

The following adverse reactions were identified during post-approval use of Campath. Because these reactions are reported voluntarily from a population of uncertain size, it is not always possible to reliably estimate their frequency or establish a causal relationship to Campath exposure. Decisions to include these reactions in labeling are typically based on one or more of the following factors: (1) seriousness of the reaction, (2) reported frequency of the reaction, or (3) strength of causal connection to Campath.

Fatal infusion reactions: [see **WARNINGS AND PRECAUTIONS (5.2)**].

Cardiovascular: congestive heart failure, cardiomyopathy, decreased ejection fraction (in patients previously treated with cardiotoxic agents).

Gastrointestinal: GI hemorrhage

Hepatic: elevation of hepatic enzymes

Immune disorders: Goodpasture's syndrome, Graves' disease, aplastic anemia, Guillain Barré syndrome, chronic inflammatory demyelinating polyradiculoneuropathy, serum sickness, Coombs test positive, transfusion associated Graft versus Host Disease.

Infections: Epstein-Barr Virus (EBV), progressive multifocal leukoencephalopathy (PML), hepatitis B virus.

Metabolic: tumor lysis syndrome, dehydration

Neoplasms: EBV-associated lymphoproliferative disorder

Neurologic: dizziness, optic neuropathy

Psychiatric: confusion

Renal: abnormal renal function

## 7 DRUG INTERACTIONS

No formal drug interaction studies have been performed with Campath.

## 8 USE IN SPECIFIC POPULATIONS

### 8.1 Pregnancy

#### Pregnancy Category C

Animal reproduction studies have not been conducted with Campath. IgG antibodies, such as Campath, can cross the placental barrier. It is not known whether Campath can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Campath should be given to a pregnant woman only if clearly needed.

### 8.3 Nursing Mothers

Excretion of Campath in human breast milk has not been studied; it is not known whether this drug is excreted in human milk. IgG antibodies, such as Campath, can be excreted in human milk. Because many drugs are excreted in human milk and because of the potential for serious adverse reactions in nursing infants from Campath, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into account the elimination half-life of Campath and the importance of the drug to the mother.

### 8.4 Pediatric Use

Safety and effectiveness have not been established in pediatric patients.

### 8.5 Geriatric Use

Of 147 previously untreated B-CLL patients treated with Campath, 35% were  $\geq$  age 65 and 4% were  $\geq$  age 75. Of 149 previously treated patients with B-CLL, 44% were  $\geq$  65 years of age and 10% were  $\geq$  75 years of age. Clinical studies of Campath did not include sufficient number of subjects age 65 and over to determine whether they respond differently than younger subjects. Other reported clinical experience has not identified differences in responses between the elderly and younger patients.

## 10 OVERDOSAGE

Across all clinical experience, the reported maximum single dose received was 90 mg. Bone marrow aplasia, infections, or severe infusions reactions occurred in patients who received a dose higher than recommended.

One patient received an 80 mg dose by IV infusion and experienced acute bronchospasm, cough, and dyspnea, followed by anuria and death. Another patient received two 90 mg doses by IV infusion one day apart during the second week of treatment and experienced a rapid onset of bone marrow aplasia.

There is no known specific antidote for Campath. Treatment of overdose consists of drug discontinuation and supportive therapy.

## 11 DESCRIPTION

Campath (alemtuzumab) is a recombinant DNA-derived humanized monoclonal antibody (Campath-1H) directed against the 21–28 kD cell surface glycoprotein, CD52. Campath-1H is an IgG1 kappa antibody with human variable framework and constant regions, and complementarity-determining regions from a murine (rat) monoclonal antibody (Campath-1G).

The Campath-1H antibody has an approximate molecular weight of 150 kD. Campath is produced in mammalian cell (Chinese hamster ovary) suspension culture in a medium containing neomycin. Neomycin is not detectable in the final product.

Campath is a sterile, clear, colorless, isotonic solution (pH 6.8–7.4) for injection. Each single use vial of Campath contains 30 mg alemtuzumab, 8.0 mg sodium chloride, 1.44 mg dibasic sodium phosphate, 0.2 mg potassium chloride, 0.2 mg monobasic potassium phosphate, 0.1 mg polysorbate 80, and 0.0187 mg disodium edetate dihydrate. No preservatives are added.

## 12 CLINICAL PHARMACOLOGY

### 12.1 Mechanism of Action

Campath binds to CD52, an antigen present on the surface of B and T lymphocytes, a majority of monocytes, macrophages, NK cells, and a subpopulation of granulocytes. A proportion of bone marrow cells, including some CD34<sup>+</sup> cells, express variable levels of CD52. The proposed mechanism of action is antibody-dependent cellular-mediated lysis following cell surface binding of Campath to the leukemic cells.

### 12.3 Pharmacokinetics

Campath pharmacokinetics were characterized in a study of 30 previously treated B-CLL patients in whom Campath was administered at the recommended dose and schedule. Campath pharmacokinetics displayed nonlinear elimination kinetics. After the last 30 mg dose, the mean volume of distribution at steady-state was 0.18 L/kg (range 0.1 to 0.4 L/kg). Systemic clearance decreased with repeated administration due to decreased receptor-mediated clearance (i.e., loss of CD52 receptors in the periphery). After 12 weeks of dosing, patients exhibited a seven-fold increase in mean AUC. Mean half-life was 11 hours (range 2 to 32 hours) after the first 30 mg dose and was 6 days (range 1 to 14 days) after the last 30 mg dose.

Comparisons of AUC in patients  $\geq$  65 years (n=6) versus patients < 65 years (n=15) suggested that no dose adjustments are necessary for age. Comparisons of AUC in female patients (n=4) versus male patients (n=17) suggested that no dose adjustments are necessary for gender.

The pharmacokinetics of Campath in pediatric patients have not been studied. The effects of renal or hepatic impairment on the pharmacokinetics of Campath have not been studied.

## 13 NONCLINICAL TOXICOLOGY

### 13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

No long-term studies in animals have been performed to establish the carcinogenic or mutagenic potential of Campath, or to determine its effects on fertility in males or females.

## 14 CLINICAL STUDIES

### 14.1 Previously Untreated B-CLL Patients

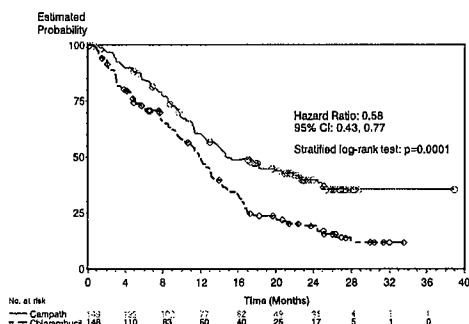
Campath was evaluated in an open-label, randomized (1:1) active-controlled study in previously untreated patients with B-CLL, Rai Stage I–IV, with evidence of progressive disease requiring therapy. Patients received either Campath 30 mg IV 3 times/week for a maximum of 12 weeks or chlorambucil 40 mg/m<sup>2</sup> PO once every 28 days, for a maximum of 12 cycles.

Of the 297 patients randomized, the median age was 60 years, 72% were male, 99% were Caucasian, 96% had a WHO performance status 0–1, 23% had maximum lymph node diameter  $\geq$  5 cm, 34% were Rai Stage III/IV, and 8% were treated in the U.S.

Patients randomized to receive Campath experienced longer progression free survival (PFS) compared to those randomized to receive chlorambucil (median PFS 14.6 months vs. 11.7 months, respectively). The overall response rates were 83% and 55% ( $p < 0.0001$ ) and the complete response rates were 24% and 2% ( $p < 0.0001$ ) for Campath and chlorambucil arms, respectively. The Kaplan-Meier curve for PFS is shown in Figure 1.

Figure 1

# Progression Free Survival in Previously Untreated B-CLL Patients<sup>1</sup>



<sup>1</sup> Log-rank test adjusted for Rai Stage (I-II vs. III-IV).

## 14.2 Previously Treated B-CLL Patients

Campath was evaluated in three multicenter, open-label, single arm studies of 149 patients with B-CLL previously treated with alkylating agents, fludarabine, or other chemotherapies. Patients were treated with the recommended dose of Campath, 30 mg intravenously, three times per week for up to 12 weeks. Partial response rates of 21 to 31% and complete response rates of 0 to 2% were observed.

## 15 REFERENCES

<sup>1</sup> American Association of Blood Banks, America's Blood Centers, American Red Cross. Circular of Information for the Use of Human Blood and Blood Components. July 2002.

## 16 HOW SUPPLIED/STORAGE AND HANDLING

Campath (alemtuzumab) is supplied in single-use clear glass vials containing 30 mg of alemtuzumab in 1 mL of solution. Each carton contains three Campath vials (NDC 50419-357-03) or one Campath vial (NDC 50419-357-01). Store Campath at 2–8°C (36–46°F). Do not freeze. If accidentally frozen, thaw at 2–8°C before administration. Protect from direct sunlight.

## 17 PATIENT COUNSELING INFORMATION

**Cytopenias:** Advise patients to report any signs or symptoms such as bleeding, easy bruising, petechiae or purpura, pallor, weakness or fatigue [see **WARNINGS AND PRECAUTIONS** (5.1) and **ADVERSE REACTIONS** (6.1)].

**Infusion Reactions:** Advise patients of the signs and symptoms of infusion reactions and of the need to take premedications as prescribed [see **WARNINGS AND PRECAUTIONS** (5.2) and **OVERALL ADVERSE REACTIONS** (6.1)].

**Infections:** Advise patients to immediately report symptoms of infection (e.g. pyrexia) and to take prophylactic anti-infectives for PCP (trimethoprim/sulfamethoxazole DS or equivalent) and for herpes virus (famciclovir or equivalent) as prescribed [see **WARNINGS AND PRECAUTIONS** (5.3) and **ADVERSE REACTIONS** (6.1)].

Advise patients that irradiation of blood products is required [see **WARNINGS AND PRECAUTIONS** (5.3)].

Advise patients that they should not be immunized with live viral vaccines if they have recently been treated with Campath [see **WARNINGS AND PRECAUTIONS** (5.5)].

Advise male and female patients with reproductive potential to use effective contraceptive methods during treatment and for a minimum of 6 months following Campath therapy [see **NONCLINICAL TOXICOLOGY** (13.1)].

U.S. Patents: 5,846,534; 6,569,430

Manufactured by:

Genzyme Corporation, Cambridge, MA 02142

Distributed by:



**Bayer HealthCare  
Pharmaceuticals**

Bayer HealthCare Pharmaceuticals Inc.  
Wayne, NJ 07470

Issued October 2008

6701102 US 81569216

**RECOMBINATE****Antihemophilic Factor****(Recombinant)****Baxter****Description**

RECOMBINATE, Antihemophilic Factor (Recombinant) (rAHF) is a glycoprotein synthesized by a genetically engineered Chinese Hamster Ovary (CHO) cell line. In culture, the CHO cell line secretes recombinant antihemophilic factor (rAHF) into the cell culture medium. The rAHF is purified from the culture medium utilizing a series of chromatography columns. A key step in the purification process is an immunoaffinity chromatography methodology in which a purification matrix, prepared by immobilization of a monoclonal antibody directed to factor VIII, is utilized to selectively isolate the rAHF in the medium. The synthesized rAHF produced by the CHO cells has the same biological effects as Antihemophilic Factor (Human) [AHF (Human)]. Structurally the protein has a similar combination of heterogenous heavy and light chains as found in AHF (Human).

RECOMBINATE rAHF is formulated as a sterile, nonpyrogenic, off-white to faint yellow, lyophilized powder preparation of concentrated recombinant AHF for intravenous injection. RECOMBINATE rAHF is available in single-dose bottles which contain nominally 250, 500 and 1000 International Units per bottle. When reconstituted with the appropriate volume of diluent, the product contains the following stabilizers in maximum amounts: 12.5 mg/mL Albumin (Human), 0.20 mg/mL calcium, 1.5 mg/mL polyethylene glycol (3350), 180 mEq/L sodium, 55 mM histidine, 1.5 µg/AHF international Unit (IU) polysorbate-80. Von Willebrand Factor (vWF) is coexpressed with the Antihemophilic Factor (Recombinant) and helps to stabilize it. The final product contains not more than 2 ng vWF/IU rAHF which will not have any clinically relevant effect in patients with von Willebrand's disease. The product contains no preservative.

Manufacturing of RECOMBINATE rAHF is shared by Baxter Healthcare Corporation and Wyeth BioPharma. The recombinant Antihemophilic Factor Concentrate (For Further Manufacturing Use), is produced by Baxter Healthcare Corporation and Wyeth BioPharma (For Further Manufacturing Use) and subsequently formulated and packaged at Baxter Healthcare Corporation.

Each bottle of RECOMBINATE rAHF is labeled with the AHF activity expressed in IU per bottle. Biological potency is determined by an *in vitro* assay which is referenced to the World Health Organization (WHO) International Standard for Factor VIII:C Concentrate.

### **Clinical Pharmacology**

AHF is the specific clotting factor deficient in patients with hemophilia A (classical hemophilia). Hemophilia A is a genetic bleeding disorder characterized by hemorrhages which may occur spontaneously or after minor trauma. The administration of RECOMBINATE rAHF provides an increase in plasma levels of AHF and can temporarily correct the coagulation defect in these patients. Pharmacokinetic studies on sixty-nine (69) patients revealed the circulating mean half-life for rAHF to be  $14.6 \pm 4.9$  hours (n=67), which was not statistically significantly different from plasma-derived **HEMOFIL M**, Antihemophilic Factor (Human) (AHF) (pdAHF). The mean half-life of **HEMOFIL M** AHF was  $14.7 \pm 5.1$  hours (n=61). The actual baseline recovery observed with rAHF was  $123.9 \pm 47.7$  IU/dl (n=23) which is significantly higher than the actual **HEMOFIL M** AHF baseline recovery of  $101.7 \pm 31.6$  IU/dl (n=61). However, the calculated ratio of actual to expected recovery with rAHF ( $121.2 \pm 48.9\%$ ) is not different on average from **HEMOFIL M** AHF ( $123.4 \pm 16.4\%$ ).

The clinical study of rAHF in previously treated patients (individuals with hemophilia A who had been treated with plasma derived AHF) was based on observations made on a study group of 69 patients. These individuals received cumulative amounts of Factor VIII ranging from 20,914 to 1,383,063 IU over the 48-month study. Patients were given a total of 17,700 infusions totaling 28,090,769 IU rAHF.



These patients were successfully treated for bleeding episodes on a demand basis and also for the prevention of bleeds (prophylaxis). Spontaneous bleeding episodes successfully managed include hemarthroses, soft tissue and muscle bleeds. Management of hemostasis was also evaluated in surgeries. A total of 24 procedures on 13 patients were performed during this study. These included minor (e.g. tooth extraction) and major (e.g. bilateral osteotomies, thoracotomy and liver transplant) procedures. Hemostasis was maintained perioperatively and postoperatively with individualized AHF replacement.

A study of rAHF in previously untreated patients was also performed as part of an ongoing study. The study group was comprised of seventy-nine (79) patients, of whom seventy-six (76) had received at least one infusion of rAHF. To date, this cohort has been given 12,209 infusions totaling over 11,277,043 IU rAHF. Hemostasis was appropriately managed in spontaneous bleeding episodes, intracranial hemorrhage and surgical procedures.

## **Indications and Usage**

The use of RECOMBINATE rAHF is indicated in hemophilia A (classical hemophilia) for the prevention and control of hemorrhagic episodes.<sup>1</sup> RECOMBINATE rAHF is also indicated in the perioperative management of patients with hemophilia A (classical hemophilia).

RECOMBINATE rAHF can be of therapeutic value in patients with acquired AHF inhibitors not exceeding 10 Bethesda Units per mL<sup>2</sup>. In clinical studies with RECOMBINATE rAHF, patients with inhibitors who were entered into the previously treated patient trial and those previously untreated children who have developed inhibitor activity on study, showed clinical hemostatic response when the titer of inhibitor was less than 10 Bethesda Units per mL. However, in such uses, the dosage of RECOMBINATE rAHF should be controlled by frequent laboratory determinations of circulating AHF levels.

RECOMBINATE rAHF is not indicated in von Willebrand's disease.

## **Contraindications**

Known hypersensitivity to mouse, hamster or bovine protein may be a contraindication to the use of Antihemophilic Factor (Recombinant) (see **Precautions**).

## **Warnings**

None.

## **Precautions**

### **General**

Certain components used in the packaging of this product contain natural rubber latex. Identification of the clotting defect as a Factor VIII deficiency is essential before the administration of RECOMBINATE, Antihemophilic Factor (Recombinant) (rAHF) is initiated. No benefit may be expected from this product in treating other deficiencies.

The formation of neutralizing antibodies, inhibitors to factor VIII, is a known complication in the management of individuals with hemophilia A. The reported prevalence of these antibodies in patients receiving plasma derived AHF is 10-20%<sup>3-7, 10-12</sup>. These inhibitors are invariably IgG immunoglobulins, the factor VIII procoagulant inhibitory activity of which is expressed as Bethesda Units (B.U.) per mL of plasma or serum<sup>3-7</sup>. Over the investigational period, none of the 69 previously treated individuals, without an inhibitor at entry into the study, developed an inhibitor. In the previously untreated patient group there were 73 eligible patients with factor VIII levels less than or equal to 2% who received at least one rAHF treatment (median days 100, range 3-821) and who were tested for inhibitor after treatment with RECOMBINATE rAHF. Of this group, 23 individuals developed detectable inhibitor (median days 10, range 3-69) and of these, 8 patients showed a titer greater than 10 B.U. Patients treated with rAHF should be carefully monitored for the development of antibodies to rAHF by appropriate clinical observations and laboratory tests.

**Formation of Antibodies to Mouse, Hamster or Bovine Protein**

As RECOMBINATE rAHF contains trace amounts of mouse protein (maximum of 0.1 ng/IU rAHF), hamster protein (maximum of 1.5 ng CHO protein/IU rAHF), and bovine protein (maximum of 1 ng BSA/IU rAHF), the remote possibility exists that patients treated with this product may develop hypersensitivity to these non-human mammalian proteins.

**Information for Patients**

The patient and physician should discuss the risks and benefits of this product.

Although allergic type hypersensitivity reactions were not observed in any patient receiving RECOMBINATE rAHF on study, such reactions are theoretically possible. Patients should be informed of the early signs of hypersensitivity reactions including hives, generalized urticaria, tightness of the chest, wheezing, hypotension, and anaphylaxis. Patients should be advised to discontinue use of the product and contact their physician if these symptoms occur.

**Laboratory Tests**

Although dosage can be estimated by the calculations which follow, it is strongly recommended that whenever possible, appropriate laboratory tests be performed on the patient's plasma at suitable intervals to assure that adequate AHF levels have been reached and are maintained.

If the patient's plasma AHF fails to reach expected levels or if bleeding is not controlled after adequate dosage, the presence of inhibitor should be suspected. By performing appropriate laboratory procedures, the presence of an inhibitor can be demonstrated and quantified in terms of AHF International Units neutralized by each mL of plasma or by the total estimated plasma volume. If the inhibitor is present at levels less than 10 Bethesda Units per mL, administration of additional AHF may neutralize the inhibitor. Thereafter, the administration of additional AHF International Units should elicit the

predicted response. The control of AHF levels by laboratory assay is necessary in this situation.

Inhibitor titers above 10 Bethesda Units per mL may make hemostasis control with AHF either impossible or impractical because of the very large dose required. In addition, the inhibitor titer may rise following AHF infusion because of an anamnestic response to the AHF antigen.

### **Carcinogenesis, Mutagenesis, Impairment of Fertility**

RECOMBINATE rAHF was tested for mutagenicity at doses considerably exceeding plasma concentrations of rAHF *in vitro* and at doses up to ten times the expected maximum clinical dose *in vivo*, and did not cause reverse mutations, chromosomal aberrations, or an increase in micronuclei in bone marrow polychromatic erythrocytes. Long term studies in animals have not been performed to evaluate carcinogenic potential.

### **Pediatric Use**

RECOMBINATE, Antihemophilic Factor (Recombinant) (rAHF) is appropriate for use in children of all ages, including the newborn. Safety and efficacy studies have been performed in both previously treated (n=23) and previously untreated (n=75) children. (See **Clinical Pharmacology and Precautions**).

### **Pregnancy**

Pregnancy Category C. Animal reproduction studies have not been conducted with Antihemophilic Factor (Recombinant). It is not known whether Antihemophilic Factor (Recombinant) can cause fetal harm when administered to a pregnant woman or can affect reproductive capacity. Antihemophilic Factor (Recombinant) should be given to a pregnant woman only if clearly needed.

### **Adverse Reactions**

During the clinical studies conducted in the previously treated patient group, there were 13 infusion related minor adverse reactions reported out of 10,446 infusions (0.12%).

One patient experienced flushing and nausea during his first infusion which abated on decreasing the infusion rate. A second patient experienced mild fatigue during and following one infusion and a third patient had a series of eleven nose bleeds with a periodicity associated with the infusions.

The protein in greatest concentration in RECOMBINATE rAHF is Albumin (Human). Reactions associated with intravenous administration of albumin are extremely rare, although nausea, fever, chills or urticaria have been reported. Other allergic reactions could theoretically be encountered in the use of this Antihemophilic Factor preparation. See **Information for Patients**.

## **Dosage and Administration**

Each bottle of RECOMBINATE rAHF is labeled with the AHF activity expressed in IU per bottle. This potency assignment is referenced to the World Health Organization International Standard for Factor VIII:C Concentrate and is evaluated by appropriate methodology to ensure accuracy of the results.

The expected *in vivo* peak increase in AHF level expressed as IU/dL of plasma or % (percent) of normal can be estimated by multiplying the dose administered per kg body weight (IU/kg) by two. This calculation is based on the clinical findings of Abildgaard *et al*<sup>8</sup> and is supported by the data generated by 419 clinical pharmacokinetic studies with rAHF in 67 patients over time. This pharmacokinetic data demonstrated a peak recovery point above the pre-infusion baseline of approximately 2.0 IU/dL per IU/kg body weight.

Example (Assuming patient's baseline AHF level is at <1%):

- (1) A dose of 1750 IU AHF administered to a 70 kg patient, i.e. 25 IU/kg (1750/70), should be expected to cause a peak post-infusion AHF increase of  $25 \times 2 = 50$  IU/dL (50% of normal).
- (2) A peak level of 70% is required in a 40 kg child. In this situation the dose would be  $70/2 \times 40 = 1400$  IU.

---

### Recommended Dosage Schedule

Physician supervision of the dosage is required. The following dosage schedule may be used as a guide.

<b>Hemorrhage</b>		
<b>Degree of hemorrhage</b>	<b>Required peak post-infusion AHF activity in the blood (as % of normal or IU/dL plasma)</b>	<b>Frequency of infusion</b>
Early hemarthrosis or muscle bleed or oral bleed	20-40	Begin infusion every 12 to 24 hours for one-three days until the bleeding episode as indicated by pain is resolved or healing is achieved.
More extensive hemarthrosis, muscle bleed, or hematoma	30-60	Repeat infusion every 12 to 24 hours for usually three days or more until pain and disability are resolved.
Life threatening bleeds such as head injury, throat bleed, severe abdominal pain	60-100	Repeat infusion every 8 to 24 hours until threat is resolved.
<b>Surgery</b>		
<b>Type of operation</b>		
Minor surgery, including tooth extraction	60-80	A single infusion plus oral antifibrinolytic therapy within one hour is sufficient in approximately 70% of cases.
Major surgery	80-100 (pre- and post-operative)	Repeat infusion every 8 to 24 hours depending on state of healing.

The careful control of the substitution therapy is especially important in cases of major surgery or life threatening hemorrhages.

Although dosage can be estimated by the calculations above, it is strongly recommended that whenever possible, appropriate laboratory tests including serial AHF assays be performed on the

patient's plasma at suitable intervals to assure that adequate AHF levels have been reached and are maintained.

Other dosage regimens have been proposed such as that of Schimpf, *et al*, which describes continuous maintenance therapy.<sup>9</sup>

**Reconstitution: Use Aseptic Technique**

1. Bring RECOMBINATE, Antihemophilic Factor (Recombinant) (rAHF) (dry concentrate) and Sterile Water for Injection, USP, (diluent) to room temperature.
2. Remove caps from concentrate and diluent bottles.
3. Cleanse stoppers with germicidal solution and allow to dry prior to use.
4. Remove protective covering from one end of double-ended needle and insert exposed needle through the center of the stopper.
5. Remove protective covering from other end of double-ended needle. Invert diluent bottle over the upright RECOMBINATE rAHF bottle, then rapidly insert free end of the needle through the RECOMBINATE rAHF bottle stopper at its center. The vacuum in the bottle will draw in the diluent.
6. Disconnect the two bottles by removing needle from diluent bottle stopper, then remove needle from RECOMBINATE rAHF bottle. Swirl gently until all material is dissolved. Be sure that RECOMBINATE rAHF is completely dissolved, otherwise active material will be removed by the filter needle.

NOTE: Do not refrigerate after reconstitution. See **Administration**.

**Administration: Use Aseptic Technique**

Administer at room temperature.

RECOMBINATE rAHF should be administered not more than 3 hours after reconstitution.

### **Intravenous Syringe Injection**

Parenteral drug products should be inspected for particulate matter and discoloration prior to administration, whenever solution and container permit. A colorless to faint yellow appearance is acceptable for RECOMBINATE rAHF.

Plastic syringes are recommended for use with this product since proteins such as AHF tend to stick to the surface of all-glass syringes.

1. Attach filter needle to a disposable syringe and draw back plunger to admit air into the syringe.
2. Insert needle into reconstituted RECOMBINATE rAHF.
3. Inject air into bottle and then withdraw the reconstituted material into the syringe.
4. Remove and discard the filter needle from the syringe; attach a suitable needle and inject intravenously as instructed under **Rate of Administration**.
5. If a patient is to receive more than one bottle of RECOMBINATE rAHF, the contents of multiple bottles may be drawn into the same syringe by drawing up each bottle through a separate unused filter needle. Filter needles are intended to filter the contents of a single bottle of RECOMBINATE rAHF only.

### **Rate of Administration**

Preparations of RECOMBINATE, Antihemophilic Factor (Recombinant) (rAHF) can be administered at a rate of up to 10 mL per minute with no significant reactions.

The pulse rate should be determined before and during administration of RECOMBINATE rAHF. Should a significant increase in pulse rate occur, reducing the rate of administration or temporarily halting the injection usually allows the symptoms to disappear promptly.

### **How Supplied**

RECOMBINATE rAHF is available in single-dose bottles which contain nominally 250, 500 and 1000 International Units per bottle. RECOMBINATE rAHF is packaged with



10 mL of Sterile Water for Injection, USP, a double-ended needle, a filter needle, one physician insert and one patient insert.

## Storage

RECOMBINATE rAHF can be stored under refrigeration [2° - 8°C (36° - 46°F)] or at room temperature, not to exceed 30°C (86°F). Avoid freezing to prevent damage to the diluent bottle. Do not use beyond the expiration date printed on the box.

## References

1. White GC, McMillan CW, Kingdon HS, *et al*: Use of recombinant antihemophilic factor in the treatment of two patients with classic hemophilia. **New Eng J Med** 320:166-170, 1989
2. Kessler CM: An Introduction to Factor VIII Inhibitors: The Detection and Quantitation. **Am J Med** 91 (Suppl 5A):1S-5S, 1991
3. Schwarzhinger I, Pabinger I, Korninger C, Haschke F, Kundi M, Niessner H, Lechner K: Incidence of inhibitors in patients with severe and moderate hemophilia A treated with factor VIII concentrates. **Am J Hematology** 24:241-245, 1987
4. Penner JA, Kelly PE: Management of patients with factor VIII or IX inhibitors. **Sem Thromb Hemostasis** 1:386-399, 1975
5. Ehrenforth S, Kreuz W, Scharer I, *et al*: Incidence of development of factor VIII and factor IX inhibitors in haemophiliacs. **Lancet** 339:594-598, 1992
6. McMillan CW, Shapiro SS, Whitehurst D, *et al*: The natural history of factor VIII inhibitors in patients with hemophilia A: a national cooperative study. II. Observations on the initial development of factor VIII:C inhibitors. **Blood** 71:344-348, 1988
7. Addiego JE Jr., Gomperts E, Liu S, *et al*: Treatment of hemophilia A with a highly purified Factor VIII concentrate prepared by Anti-FVIIIc immunoaffinity chromatography. **Thrombosis and Haemostasis** 67:19-27, 1992
8. Abildgaard CF, Simone JV, Corrigan JJ, *et al*: Treatment of hemophilia with glycine-precipitated Factor VIII. **New Eng J Med** 275:471-475, 1966

9. Schimpf K, Rothman P, Zimmermann K: Factor VIII dosis in prophylaxis of hemophilia A; A further controlled study in **Proc XIth Cong W.F.H.** Kyoto, Japan, Academic Press, 1976, pp 363-366
10. Gill FM: The Natural History of Factor VIII Inhibitors in Patients with Hemophilia A. Hoyer LW (ed), Factor VIII Inhibitors, **N.Y. AR Liss**, 1984, pp 19-29
11. Rasi V, Ikkala E: Haemophiliacs with factor VIII inhibitors in Finland: prevalence, incidence and outcome. **Br J Haematol** 76:369-371, 1990
12. Lusher JM, Salzman PM: Viral Safety and Inhibitor Development Associated with Factor VIII C Ultra-Purified From Plasma in Hemophiliacs Previously Unexposed to Factor VIII C Concentrates. **Seminars in Hematology** 27:1-7, 1990

To enroll in the confidential, industry-wide Patient Notification System, call 1-888-UPDATE U (1-888-873-2838).

Baxter, Recombinate, and Hemofil are trademarks of Baxter International, Inc.  
Baxter and Hemofil are registered in the U.S. Patent and Trademark office.

© Copyright 1992, 2004 Baxter Healthcare Corporation. All rights reserved.

Manufactured by:  
**Baxter Healthcare Corporation**  
Westlake Village, CA 91362 USA  
U.S. License No. 140  
Printed in USA

Imported in Canada by:  
**Baxter Corporation**  
Toronto, Ontario  
  
Revised December 2004









## GAMMAGARD LIQUID [Immune Globulin Intravenous (Human)] 10%

### DESCRIPTION

GAMMAGARD LIQUID Immune Globulin Intravenous (Human), 10% is a ready-for-use sterile, liquid preparation of highly purified and concentrated immunoglobulin G (IgG) antibodies. The distribution of the IgG subclasses is similar to that of normal plasma.<sup>1,2</sup> The Fc and Fab functions are maintained in GAMMAGARD LIQUID. Pre-kallikrein activator activity is not detectable. GAMMAGARD LIQUID contains 100 mg/mL protein. At least 98% of the protein is gammaglobulin, the average immunoglobulin A (IgA) concentration is 37 µg/mL, and immunoglobulin M is present in trace amounts. GAMMAGARD LIQUID contains a broad spectrum of IgG antibodies against bacterial and viral agents. Glycine (0.25M) serves as a stabilizing and buffering agent, and there are no added sugars, sodium or preservatives. The pH is 4.6 to 5.1. The osmolality is 240-300 mOsmol/kg, which is similar to physiological osmolality (285 to 295 mOsmol/kg).<sup>3</sup>

GAMMAGARD LIQUID is manufactured from large pools of human plasma. Screening against potentially infectious agents begins with the donor selection process and continues throughout plasma collection and plasma preparation. Each individual plasma donation used in the manufacture of GAMMAGARD LIQUID is collected only at FDA approved blood establishments and is tested by FDA licensed serological tests for Hepatitis B Surface Antigen (HBsAg), and for antibodies to Human Immunodeficiency Virus (HIV-1/HIV-2) and Hepatitis C Virus (HCV) in accordance with U.S. regulatory requirements. As an additional safety measure, mini-pools of the plasma are tested for the presence of HIV-1 and HCV by FDA licensed Nucleic Acid Testing (NAT) and found negative. IgGs are purified from plasma pools using a modified Cohn-Oncley cold ethanol fractionation process, as well as cation and anion exchange chromatography.

To further improve the margin of safety, three dedicated, independent and effective virus inactivation/removal steps have been integrated into the manufacturing and formulation processes, namely solvent/detergent (S/D) treatment,<sup>4,5</sup> 35 nm nanofiltration,<sup>6,7</sup> and a low pH incubation at elevated temperature.<sup>8,9</sup> The S/D process includes treatment with an organic mixture of tri-n-butyl phosphate, octoxynol 9 and polysorbate 80 at 18°C to 25°C for a minimum of 60 minutes.

*In vitro* virus spiking studies have been used to validate the capability of the manufacturing process to inactivate and remove viruses. To establish the minimum applicable virus clearance capacity of the manufacturing process, these virus clearance studies were performed under extreme conditions (e.g., at minimum S/D concentrations, incubation time and temperature for the S/D treatment). Virus clearance studies for GAMMAGARD LIQUID performed in accordance with good laboratory practices (Table 1) have demonstrated that:

- S/D treatment inactivates the lipid-enveloped viruses investigated to below detection limits within minutes.
- 35 nm nanofiltration removes lipid-enveloped viruses to below detection limits and reduces the non-lipid enveloped viruses HAV and B19V. As determined by a polymerase chain reaction assay, nanofiltration reduced B19V by a mean log<sub>10</sub> reduction factor of 4.8 genome equivalents.
- Treatment with low pH at elevated temperature of 30°C to 32°C inactivates lipid-enveloped viruses and encephalomyocarditis virus (EMCV, model for HAV) to below detection limits, and reduces mice minute virus (MMV, model for B19V).

Virus type Family	Enveloped RNA			Enveloped DNA	Non-enveloped RNA			Non-enveloped DNA
	Retroviridae	Flaviviridae	Herpesviridae	Picomaviridae	Parvoviridae			
Virus	HIV-1	BVDV	WNV	PRV	HAV	EMCV	MMV	
SD treatment	>4.5	>6.2	n.a.	>4.8	n.d.	n.d.	n.d.	
35 nm nanofiltration	>4.5	>5.1	>6.2	>5.6	5.7	1.4	2.0	
Low pH treatment	>5.8	>5.5	>6.0	>6.5	n.d. <sup>b</sup>	>6.3	3.1	
Overall log reduction factor (ORF)	>14.8	>16.8	>12.2	>16.9	5.7 <sup>b</sup>	>7.7	5.1	

Abbreviations: HIV-1, Human Immunodeficiency Virus Type 1; BVDV, Bovine Viral Diarrhea Virus (model for Hepatitis C Virus and other lipid enveloped RNA viruses); WNV, West Nile Virus; PRV, Pseudorabies Virus (model for lipid enveloped DNA viruses, including Hepatitis B Virus); EMCV, Encephalomyocarditis Virus (model for non-lipid enveloped RNA viruses, including Hepatitis A virus [HAV]); MMV, Mice Minute Virus (model for non-lipid enveloped DNA viruses, including B19 virus [B19V]); n.d. (not done), n.a. (not applicable).

<sup>a</sup> For the calculation of these RF data from virus clearance study reports, applicable manufacturing conditions were used. Log<sub>10</sub> RFs on the order of 4 or more are considered effective for virus clearance in accordance with the Committee for Medicinal Products for Human Use (CHMP, formerly CPMP) guidelines.

<sup>b</sup> No RF obtained due to immediate neutralization of HAV by the anti-HAV antibodies present in the product.

### CLINICAL PHARMACOLOGY

#### Clinical Efficacy

Use of GAMMAGARD LIQUID in patients with Primary Immunodeficiency is supported by the Phase 3 clinical study of subjects who were treated with 300 to 600 mg/kg every 21 to 28 days for 12 months. The 61 subjects in this study were between 6 to 72 years of age, 54% female and 46% male, and 93% Caucasian, 5% African-American, and 2% Asian. Three subjects were excluded from the per-protocol analysis due to non-study product related reasons. The primary efficacy endpoint was the annualized rate of specified acute serious bacterial infections, i.e., the mean number of specified acute serious bacterial infections per subject per year (see Table 2).

Table 2: Summary of Validated Acute Serious Bacterial Infections for the Per-Protocol Analysis

	Number of Events
Validated Infections*	
Bacteremia / Sepsis	0
Bacterial Meningitis	0
Osteomyelitis / Septic Arthritis	0
Bacterial Pneumonia	0
Visceral Abscess	0
Total	0
Hospitalizations Secondary to Infection	0
Mean Number of Validated Infections per Subject per Year	0
p-value*	p < 0.0001
95% Confidence Interval*	(0.000, 0.064)

<sup>a</sup> Serious acute bacterial infections were defined by FDA and met specific diagnostic requirements.

<sup>b</sup> The rate of validated infections was compared with a rate of 1 per subject per year, in accordance with recommendations by the FDA Blood Products Advisory Committee.<sup>10</sup>

The secondary efficacy endpoints in this study were the annualized rate of other specified validated bacterial infections (see Table 3), and the number of hospitalizations secondary to all validated infectious complications (see Table 2 and Table 3).

Table 3: Summary of Validated Other Bacterial Infections

	Number of Events
Validated Infections*	
Urinary Tract Infection	1
Gastroenteritis	1
Lower Respiratory Tract Infection: Tracheobronchitis, Bronchiolitis Without Evidence of Pneumonia	0
Lower Respiratory Tract Infection: Other Infections (e.g., Lung Abscess, Empyema)	0
Otitis Media	2
Total	4
Hospitalizations Secondary to Infection	0
Mean Number of Validated Infections per Subject per Year	0.07
95% Confidence Interval	(0.018, 0.168)

<sup>a</sup> Other bacterial infections that met specific diagnostic requirements.

In this study, there were no validated acute serious bacterial infections in any of the treated subjects. The annualized rate of acute serious bacterial infections was significantly less than (p < 0.0001) the rate of one infection per year, in accordance with recommendations by the FDA Blood Products Advisory Committee.<sup>10</sup> Four of the 61 subjects reported a total of 4 other specified validated bacterial infections. None were serious or severe, none resulted in hospitalization, and all resolved completely.

The rate of all clinically-defined but non-validated infections was 3.4 infections per patient per year. These consisted primarily of recurrent episodes of commonly observed infections in this patient population - sinusitis, bronchitis, nasopharyngitis, urinary tract infections, and upper respiratory infections.

#### Pharmacokinetics

The overall pharmacokinetic characteristics of Immune Globulin Intravenous (Human) [IGIV] products are well-described in the literature.<sup>11,12</sup> Following infusion, IGIV products show a biphasic decay curve. The initial (α) phase is characterized by an immediate post-infusion peak in serum IgG and is followed by rapid decay due to equilibration between the plasma and extravascular fluid compartments. The second (β) phase is characterized by a slower and constant rate of decay.

The commonly cited "normal" half life of 18 to 25 days is based on studies in which tiny quantities of radiolabeled IgG are injected into healthy individuals.<sup>13,14</sup> When radiolabeled IgG was injected into patients with hypogammaglobulinemia or agammaglobulinemia, highly variable half-lives ranging from 12 to 40 days were observed.<sup>13,14</sup> In other radiolabeled studies, high serum concentrations of IgG, and hypermetabolism associated with fever and infection, have been seen to coincide with a shortened half-life of IgG.<sup>14,15,16,17</sup>

In contrast, however, pharmacokinetic studies in immunodeficient patients are based on the decline of IgG concentrations following infusions of large quantities of gammaglobulin. In such trials, investigators have reported uniformly prolonged half-lives of 26 – 35 days.<sup>16,18,19,20,21,22</sup>

Pharmacokinetic parameters for GAMMAGARD LIQUID were determined from total IgG levels following the fourth infusion. A total of 61 subjects were enrolled and treated. Of these, 57 had sufficient pharmacokinetic data to be included in the dataset. Pharmacokinetic parameters are presented in Table 4.

Table 4: Summary of Pharmacokinetic Parameters in 57 Subjects

Parameter	Median	95% Confidence Interval
Elimination Half-Life (T <sub>1/2</sub> days)	35	(31, 42)
AUC <sub>0-24</sub> (mg-days/dL)	29139	(27494, 30490)
C <sub>max</sub> (Peak, mg/dL)	2050	(1980, 2200)
C <sub>min</sub> (Trough, mg/dL)	1030	(939, 1110)
Incremental recovery (mg/dL)/(mg/kg)	2.3	(2.2, 2.6)

Abbreviations: AUC= area under the curve;

C<sub>max</sub>=maximum concentration; C<sub>min</sub>= minimum concentration



Median IgG trough levels were maintained between 960-1120 mg/dL. These dosing regimens maintained serum trough IgG levels considerably above 450 mg/dL, which is consistent with levels considered to be effective in the treatment of patients with Primary Immunodeficiency.<sup>23,24</sup> The elimination half-life of GAMMAGARD LIQUID of 35 days was similar to the half-lives reported for other IGIV products.<sup>1,3,14,15,17,25,26</sup>

## INDICATIONS AND USAGE

### Primary Immunodeficiency

GAMMAGARD LIQUID is indicated for the treatment of primary immunodeficiency disorders associated with defects in humoral immunity. These include but are not limited to congenital X-linked agammaglobulinemia, common variable immunodeficiency, Wiskott-Aldrich syndrome, and severe combined immunodeficiencies.<sup>15,22</sup>

## CONTRAINDICATIONS

GAMMAGARD LIQUID is contraindicated in patients with known anaphylactic or severe hypersensitivity responses to Immune Globulin (Human).

Patients with severe selective IgA deficiency (IgA < 0.05 g/L) may develop anti-IgA antibodies that can result in a severe anaphylactic reaction. Anaphylaxis can occur using GAMMAGARD LIQUID even though it contains low amounts of IgA (average concentration of 37 µg/mL). These patients should be treated only if their IgA deficiency is associated with an immune deficiency for which therapy with intravenous immune globulin is clearly indicated. Such patients should only receive intravenous immune globulin with utmost caution and in a setting where supportive care is available for treating life-threatening reactions.

## WARNINGS

Immune Globulin Intravenous (Human) products have been reported to be associated with renal dysfunction, acute renal failure, osmotic nephrosis, and death.<sup>27</sup> Patients predisposed to acute renal failure include patients with any degree of pre-existing renal insufficiency, diabetes mellitus, age greater than 65, volume depletion, sepsis, paraproteinemia, or patients receiving known nephrotoxic drugs. Especially in such patients, IGIV products should be administered at the minimum concentration available and the minimum rate of infusion practicable. While these reports of renal dysfunction and acute renal failure have been associated with the use of many of the licensed IGIV products, those containing sucrose as a stabilizer accounted for a disproportionate share of the total number.

Glycine, an amino acid, is used as a stabilizer. GAMMAGARD LIQUID does not contain sucrose.

See PRECAUTIONS and DOSAGE AND ADMINISTRATION sections for important information intended to reduce the risk of acute renal failure.

Immune Globulin Intravenous (Human), 10% is made from human plasma. Products made from human plasma may contain infectious agents, such as viruses, that can cause disease. The risk that such products will transmit an infectious agent has been reduced by screening plasma donors for prior exposure to certain viruses, by testing for the presence of certain current virus infections, and by inactivating and/or removing certain viruses (see DESCRIPTION). Despite these measures, such products can still potentially transmit disease. Because this product is made from human blood, it may carry a risk of transmitting infectious agents, e.g., viruses and theoretically, the Creutzfeldt-Jakob disease (CJD) agent. ALL infections thought by a physician possibly to have been transmitted by this product should be reported by the physician or other healthcare provider to Baxter Healthcare Corporation, at 1-800-423-2862 (in the U.S.). The physician should discuss the risks and benefits of this product with the patient.

GAMMAGARD LIQUID should only be administered intravenously. Other routes of administration have not been evaluated.

Immediate anaphylactic and hypersensitivity reactions are a remote possibility. Epinephrine and antihistamines should be available for treatment of any acute anaphylactoid reactions.

## PRECAUTIONS

### General

Some viruses, such as B19V (formerly known as Parvovirus B19) or Hepatitis A, are particularly difficult to remove or inactivate. B19V most seriously affects pregnant women, or immune-compromised individuals. Symptoms of B19V infection include fever, drowsiness, chills and runny nose followed about two weeks later by a rash and joint pain. Evidence of Hepatitis A may include several days to weeks of poor appetite, tiredness, and low-grade fever followed by nausea, vomiting and abdominal pain. Dark urine and a yellowed complexion are also common symptoms. Patients should be encouraged to consult their physician if such symptoms appear.

Components used in the packaging of this product are latex-free.

### Renal Function

Periodic monitoring of renal function tests and urine output is particularly important in patients judged to have a potential increased risk for developing acute renal failure. Assume that patients are not volume depleted prior to the initiation of infusion of GAMMAGARD LIQUID. Renal function, including measurement of blood urea nitrogen (BUN)/serum creatinine, should be assessed prior to the initial infusion of IGIV products and again at appropriate intervals thereafter. If renal function deteriorates, discontinuation of the product should be considered.

For patients judged to be at risk of developing renal dysfunction, it may be prudent to reduce the rate of infusion to less than 3.3 mg IgG/kg/min (<2 mL/kg/hr).

### Hemolysis

IGIV products can contain blood group antibodies which may act as hemolysins and induce *in vivo* coating of red blood cells with immunoglobulin, causing a positive direct antiglobulin reaction and, rarely, hemolysis.<sup>28,29,30</sup> Hemolytic anemia can develop subsequent to IGIV therapy due to enhanced red blood cells (RBC) sequestration (see ADVERSE REACTIONS).<sup>31</sup> IGIV recipients should be monitored for clinical signs and symptoms of hemolysis (see PRECAUTIONS: Laboratory Tests).

## Transfusion-Related Acute Lung Injury (TRALI)

There have been reports of noncardiogenic pulmonary edema (Transfusion Related Acute Lung Injury [TRALI]) in patients administered IGIV.<sup>32</sup> TRALI is characterized by severe respiratory distress, pulmonary edema, hypoxemia, normal left ventricular function, and fever, and typically occurs within 1-6 hours after transfusion. Patients with TRALI may be managed using oxygen therapy with adequate ventilatory support.

IGIV recipients should be monitored for pulmonary adverse reactions. If TRALI is suspected, appropriate tests should be performed for the presence of anti-neutrophil antibodies in both the product and patient serum (see PRECAUTIONS: Laboratory Tests).

## Thrombotic Events

Thrombotic events have been reported in association with IGIV (see ADVERSE REACTIONS).<sup>33,34,35,36,37,38,39,40,41</sup> Patients at risk may include those with a history of atherosclerosis, multiple cardiovascular risk factors, advanced age, impaired cardiac output, and/or known or suspected hyperviscosity, hypercoagulable disorders and prolonged periods of immobilization. The potential risks and benefits of IGIV should be weighed against those of alternative therapies for all patients for whom IGIV administration is being considered. Baseline assessment of blood viscosity should be considered in patients at risk for hyperviscosity, including those with cryoglobulins, fasting chylomicronemia/markedly high triacylglycerols (triglycerides), or monoclonal gammopathies (see PRECAUTIONS: Laboratory Tests).

## Aseptic Meningitis Syndrome

An aseptic meningitis syndrome (AMS) has been reported to occur infrequently in association with IGIV treatment. Discontinuation of IGIV treatment has resulted in remission of AMS within several days without sequelae. The syndrome usually begins within several hours to two days following IGIV treatment. It is characterized by symptoms and signs including severe headache, nuchal rigidity, drowsiness, fever, photophobia, painful eye movements, and nausea and vomiting. Cerebrospinal fluid (CSF) studies are frequently positive with pleocytosis up to several thousand cells per cubic mm, predominantly from the granulocytic series, and elevated protein levels up to several hundred mg/dL. Patients exhibiting such symptoms and signs should receive a thorough neurological examination, including CSF studies, to rule out other causes of meningitis. AMS may occur more frequently in association with high dose (2 g/kg) IGIV treatment.

## Laboratory Tests

If signs and/or symptoms of hemolysis are present after IGIV infusion, appropriate confirmatory laboratory testing should be done [see PRECAUTIONS].

If TRALI is suspected, appropriate tests should be performed for the presence of anti-neutrophil antibodies in both the product and patient serum [see PRECAUTIONS].

Because of the potentially increased risk of thrombosis, baseline assessment of blood viscosity should be considered in patients at risk for hyperviscosity, including those with cryoglobulins, fasting chylomicronemia/markedly high triacylglycerols (triglycerides), or monoclonal gammopathies [see PRECAUTIONS].

## Information For Patients

Patients should be instructed to immediately report symptoms of decreased urine output, sudden weight gain, fluid retention/edema, and/or shortness of breath (which may suggest kidney damage) to their physicians.

## Drug Interactions

See DOSAGE AND ADMINISTRATION section.

## Pregnancy Category C

Animal reproduction studies have not been conducted with GAMMAGARD LIQUID. It is also not known whether GAMMAGARD LIQUID can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. GAMMAGARD LIQUID should be given to a pregnant woman only if clearly indicated. Maternally administered IGIV products have been shown to cross the placenta, increasingly after 30 weeks gestation.<sup>42,43,44</sup>

## Use in Pediatrics

The safety and efficacy of GAMMAGARD LIQUID has not been evaluated in neonates or infants.

## ADVERSE REACTIONS

### General

Various mild and moderate reactions, such as headache, fever, fatigue, chills, flushing, dizziness, urticaria, wheezing or chest tightness, nausea, vomiting, rigors, back pain, chest pain, muscle cramps, and changes in blood pressure may occur with infusions of Immune Globulin Intravenous (Human). In general, reported adverse reactions to GAMMAGARD LIQUID in patients with Primary Immunodeficiency are similar in kind and frequency to those observed with other IGIV products. Slowing or stopping the infusion usually allows the symptoms to disappear promptly. Although hypersensitivity reactions have not been reported in the clinical studies with GAMMAGARD LIQUID immediate anaphylactic and hypersensitivity reactions are a remote possibility. Epinephrine and antihistamines should be available for treatment of any acute anaphylactic reactions (see WARNINGS).

## Clinical Study

Adverse experiences were examined among a total of 61 enrolled subjects with Primary Immunodeficiency who received at least one infusion of GAMMAGARD LIQUID during the Phase 3 multicenter clinical study. For this study, temporally associated adverse events are defined by the FDA as those occurring during or within 72 hours of completion of an infusion. Adverse drug reactions (ADRs) are those adverse events that were deemed by the investigators as causally related to the infusion of GAMMAGARD LIQUID.

**Baxter**



Of all adverse experiences, 15 events in 8 subjects were serious. Two serious events, two episodes of aseptic meningitis in one patient, were deemed to be possibly related to the infusion of GAMMAGARD LIQUID.

Among the 896 non-serious adverse experiences, 258 were judged by the investigator to be possibly or probably related to the infusion of GAMMAGARD LIQUID. Of these, 136 were mild, 106 were moderate, and 16 were severe. All of the severe non-serious adverse experiences were transient, did not lead to hospitalization, and resolved without complication. One subject withdrew from the study due to a non-serious adverse experience (papular rash).

Of the 345 temporally related adverse experiences, those occurring in > 5% of subjects are shown in Table 5. Of these events, only headache occurred in association with more than 5% of infusions. All events were expected based on past experiences with intravenous gammaglobulin products.

Table 5: Adverse Events*, Regardless of Causality, that Occurred within 72 Hours of Infusion				
Event	By Infusion		By Subject	
	Number	Percentage	Number	Percentage
Headache	57	6.90	22	36.1
Fever	19	2.30	13	21.3
Fatigue	18	2.18	10	16.4
Vomiting	10	1.21	9	14.8
Chills	14	1.69	8	13.1
Infusion site events	8	0.97	8	13.1
Nausea	9	1.09	6	9.8
Dizziness	7	0.85	6	9.8
Pain in Extremity	7	0.85	5	8.2
Diarrhea	7	0.85	5	8.2
Cough	5	0.61	5	8.2
Pruritus	5	0.61	4	6.5
Pharyngeal Pain	5	0.61	4	6.5

\* Excluding Infections

The majority (227/258) of the non-serious adverse experiences deemed related to study product were considered expected based on previous experience with IGIV products and 31 were considered unexpected. In virtually every case, these unexpected events were either consistent with the subject's specific type of immunodeficiency or with the subject's medical history prior to entering the study. A total of 14 hospitalizations occurred during the study but none were related to infection.

Hematology and clinical chemistry parameters were monitored in all subjects prior to each infusion throughout the 12-month period of study. Mean values for all laboratory parameters remained consistent throughout the study period. Three of the hematology values in one subject were outside of the normal range and reported as non-serious adverse experiences that resolved completely. These were a red cell count of  $3.9 \times 10^6/\mu\text{L}$ , hematocrit of 31%, and white cell count of  $3.88 \times 10^3/\mu\text{L}$ . All spontaneously returned to baseline. One subject had an elevated BUN (45 mg/dL) and creatinine (1.4 mg/dL) on one occasion that were reported as non-serious adverse experiences and resolved completely. These values improved to 30 mg/dL and 0.8 mg/dL, respectively, by the next infusion. Six of the patients had a single, transient elevation in serum transaminases. Two additional patients had persistent elevations in transaminases, ALT and AST, which were present at the initiation of the study, prior to the infusion of GAMMAGARD LIQUID. There was no other evidence of liver abnormalities. None of the hematology or chemistry laboratory abnormalities that occurred during the course of the study required clinical intervention and none had clinical consequences.

During the Phase 3 clinical study, viral safety was assessed by serological screening for HBsAg and antibodies to HCV and HIV-1 and HIV-2 prior to, during, and at the end of the study and by Polymerase Chain Reaction (PCR) tests for HBV, HCV, and HIV-1 genomic sequences prior to and at the end of the study. None of the 61 treated subjects were positive prior to study entry and none converted from negative to positive during the 12-month period of study.

#### Postmarketing:

The following is a list of adverse reactions that have been identified and reported during the post-approval use of IGIV products:

<b>Respiratory</b> cyanosis, hypoxemia, pulmonary edema, dyspnea, bronchospasm
<b>Cardiovascular</b> thromboembolism, hypotension
<b>Neurological</b> seizures, tremor
<b>Hematologic</b> hemolysis, positive direct antiglobulin (Coombs) test
<b>General/Body as a Whole</b> pyrexia, rigors
<b>Musculoskeletal</b> back pain
<b>Gastrointestinal</b> hepatic dysfunction, abdominal pain

#### Rare and Uncommon Adverse Events:

<b>Respiratory</b> apnea, Acute Respiratory Distress Syndrome (ARDS), Transfusion Related Acute Lung Injury (TRALI)
<b>Integumentary</b> bullous dermatitis, epidermolysis, erythema multiforme, Stevens-Johnson syndrome
<b>Cardiovascular</b> cardiac arrest, vascular collapse
<b>Neurological</b> coma, loss of consciousness
<b>Hematologic</b> pancytopenia, leukopenia

Because postmarketing reporting of these reactions is voluntary and the at-risk populations are of uncertain size, it is not always possible to reliably estimate the frequency of the reaction to establish a causal relationship to exposure to the product. Such is also the case with literature reports authored independently\* (see PRECAUTIONS).

#### DOSAGE AND ADMINISTRATION

GAMMAGARD LIQUID should be at room temperature during administration.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration. Do not use if particulate matter and/or discoloration is observed. Only clear or slightly opalescent and colorless or pale yellow solutions are to be administered. GAMMAGARD LIQUID should only be administered intravenously. Other routes of administration have not been evaluated. The use of an in-line filter is optional.

For patients with Primary Immunodeficiency, monthly doses of approximately 300 – 600 mg/kg infused at 3 to 4 week intervals are commonly used.<sup>23,24</sup> As there are significant differences in the half-life of IgG among patients with Primary Immunodeficiency, the frequency and amount of immunoglobulin therapy may vary from patient to patient. The proper amount can be determined by monitoring clinical response. The minimum serum concentration of IgG necessary for protection varies among patients and has not been established by controlled clinical studies.

#### Rate of Administration

During the first infusion of the Phase 3 clinical study, GAMMAGARD LIQUID was infused at an initial rate of 0.5 mL/kg/hr (0.8 mg/kg/min). The rate was gradually increased every 30 minutes to a rate of 5.0 mL/kg/hr (8.9 mg/kg/min) if it was well tolerated. However, some patients completed the infusion before the maximum rate could be obtained. During subsequent infusions the initial rate and the rate of escalation were based on their previous infusion history; however, the maximum rate attained during the first infusion was used throughout the remainder of the study. The mean rate attained by all patients was 4.3 mL/kg/hr. Fifty-eight subjects (95%) achieved a maximum rate of 4.0 mL/kg/hr or greater and of these, 16 subjects (26%) attained a rate of 5.0 mL/kg/hr.

In general, it is recommended that patients beginning therapy with IGIV or switching from one IGIV product to another be started at the lower rates and then advanced to the maximal rate if they have tolerated several infusions at intermediate rates of infusion. It is important to individualize rates for each patient.

As noted in the WARNINGS section, **patients who have underlying renal disease or who are judged to be at risk of developing thrombotic events should not be infused rapidly with any IGIV product.** Although there are no prospective studies demonstrating that any concentration or rate of infusion is completely safe, it is believed that risk is decreased at lower rates of infusion.<sup>46</sup> Therefore, as a guideline, it is recommended that these patients who are judged to be at risk of renal dysfunction or thrombotic complications be gradually titrated up to a more conservative maximal rate of less than 3.3 mgIgG/kg/min (< 2mL/kg/hr).

A rate of administration that is too rapid may cause flushing and changes in pulse rate and blood pressure. Slowing or stopping the infusion usually results in the prompt disappearance of signs. The infusion may then be resumed at a rate that is comfortable for the patient.

#### Drug Interactions

Antibodies in IGIV products may interfere with patient responses to live vaccines, such as those for measles, mumps and rubella.<sup>47,48,49</sup> The immunizing physician should be informed of recent therapy with IGIV products so that appropriate precautions can be taken.

Admixtures of GAMMAGARD LIQUID with other drugs and intravenous solutions have not been evaluated. It is recommended that GAMMAGARD LIQUID be administered separately from other drugs or medications that the patient may be receiving. The product should not be mixed with IGIV products from other manufacturers.

Normal saline should not be used as a diluent. If dilution is preferred, GAMMAGARD LIQUID may be diluted with 5% dextrose in water (DSW).<sup>50</sup> No other drug interactions or compatibilities have been evaluated.

#### HOW SUPPLIED

GAMMAGARD LIQUID is supplied in single use bottles as follows:

NDC Number	Volume	Grams
0944-2700-02	10 mL	1.0
0944-2700-03	25 mL	2.5
0944-2700-04	50 mL	5.0
0944-2700-05	100 mL	10.0
0944-2700-06	200 mL	20.0

**Baxter**

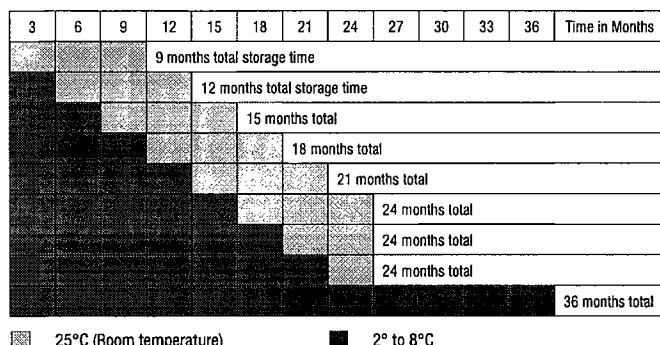
## STORAGE

**Refrigeration:** 36 months storage at refrigerated temperature 2° to 8°C (36°-46°F). Do not freeze.

**Room Temperature:** 9 months storage at room temperature 25°C, (77°F) within the first 24 months of the date of manufacture. See below for detailed storage information.

The total storage time of GAMMAGARD LIQUID depends on the point of time the vial is transferred to room temperature. Examples for total storage times are illustrated in Figure 1. The new expiration date must be recorded on the package when the product is transferred to room temperature.

Figure 1: Storage Guidelines  
Months from Date of Manufacture



## Storage Details:

- Example 1: If the product is taken out of the refrigerator after 3 months from date of manufacture, it can be stored for 9 months at room temperature. Total storage time is 12 months.
- Example 2: If the product is taken out of the refrigerator after 21 months from the date of manufacture, it can be stored for 3 additional months at room temperature. Total storage time is 24 months.
- After 24 months from date of manufacture, product cannot be stored at room temperature.

To enroll in the confidential, industry-wide Patient Notification System, call 1-888-UPDATE U (1-888-873-2838)

BAXTER and GAMMAGARD LIQUID are trademarks of Baxter International Inc.

**Baxter Healthcare Corporation**  
Westlake Village, CA 91362 USA  
U.S. License No. 140

LE-07-01130

April 2005

## REFERENCES

- Skvaril F. Qualitative and quantitative aspects of IgG subclasses in i.v. immunoglobulin preparations. In: Nydegger UE, ed. Immunotherapy. London: Academic Press; 1981:118-122.
- French M. Serum IgG subclasses in normal adults. Monogr Allergy. 1986;19:100-107.
- Lacy CF, Armstrong LL, Goldman MP, Lance LL. Appendix: Abbreviations and Measurements. Drug Information Handbook. Lexi-Comp; 1999:1254.
- Horowitz B, Prince AM, Hamman J, Watkevich C. Viral safety of solvent/detergent-treated blood products. Blood Coagul Fibrinolysis. 1994;5 Suppl 3:S21-S28.
- Kreil TR, Berling A, Kistner O, Kindermann J. West Nile virus and the safety of plasma derivatives: verification of high safety margins, and the validity of predictions based on model virus data. Transfusion. 2003;43:1023-1028.
- Hamamoto Y, Harada S, Kobayashi S, et al. A novel method for removal of human immunodeficiency virus: filtration with porous polymeric membranes. Vox Sang. 1989;56:230-236.
- Yusa T, Ishikawa G, Manabe S, Sekiguchi S, Takeuchi K, Miyamura T. The particle size of hepatitis C virus estimated by filtration through microporous regenerated cellulose fibre. J Gen Virol. 1991;72 (Pt 8):2021-2024.
- Kempf C, Jentsch P, Poirier B, et al. Virus inactivation during production of intravenous immunoglobulin. Transfusion. 1991;31:423-427.
- Louie RE, Galloway CJ, Dumas ML, Wong MF, Mitra G. Inactivation of hepatitis C virus in low pH intravenous immunoglobulin. Biologics. 1994;22:13-19.
- Golding B. IGIV Clinical Endpoints. Presented at: Blood Products Advisory Committee, 65th Meeting. 17 March 2000. Silver Spring, MD.
- Schiff RL. Intravenous immunoglobulins for treatment of antibody deficiencies. In: Good RA, Lindenlaub E, eds. The Nature, Cellular, and Biochemical Basis and Management of Immunodeficiencies. Symposium Vermed, West Germany. 21-25 September. Stuttgart: F. K. Schattauer Verlag; 1986:523-541.
- Morell A. Pharmacokinetics of intravenous immunoglobulin preparations. In: Lee ML, Strand V, eds. Intravenous Immunoglobulins in Clinical Practice. New York: M. Dekker, Inc.; 1997:1-18.
- Morell A, Skvaril F. Structure and Biological Properties of Immunoglobulins and γ-Globulin Preparations. II. Properties of γ-Globulin Preparations. Schweizerische Medizinische Wochenschrift 1980; 110:80-85.
- Waldmann TA, Strober W. Metabolism of immunoglobulins. Prog Allergy. 1969;13:1-110.
- Stiehm ER. Standard and special human immune serum globulins as therapeutic agents. Pediatrics. 1979;63:301-319.
- Lee ML, Mankarious S, Ochs H, Fischer S, Wedgwood RJ. The pharmacokinetics of total IgG, IgG subclasses, and type specific antibodies in immunodeficient patients. Immunol Invest. 1991;20:193-198.
- Buckley RH. Immunoglobulin replacement therapy: indications and contraindications for use and variable IgG levels achieved. In: Alving BM, Finlayson JS, eds. Immunoglobulins: characteristics and use of intravenous preparations. Washington, D.C.: US Department of Health and Human Services; 1979:3-8.
- Mankarious S, Lee M, Fischer S, et al. The half-lives of IgG subclasses and specific antibodies in patients with primary immunodeficiency who are receiving intravenously administered immunoglobulin. J Lab Clin Med. 1988;112:634-640.
- Pirofsky B. Safety and toxicity of a new serum immunoglobulin G Intravenous preparation, IGIV pH 4.25. Rev Infect Dis. 1986;8 Suppl 4:S457-63.
- Pirofsky B. Clinical use of a new pH 4.25 intravenous immunoglobulin preparation (Gamimune-N). J Infect. 1987;15 Suppl 1:29-37.
- Schiff RL. Half-life and clearance of pH 6.8 and pH 4.25 immunoglobulin G Intravenous preparations in patients with primary disorders of humoral immunity. Rev Infect Dis. 1986;8 Suppl 4:S449-56.
- Schiff RL, Rudd C. Alterations in the half-life and clearance of IgG during therapy with intravenous gamma-globulin in 18 patients with severe primary humoral immunodeficiency. J Clin Immunol. 1986;6:256-264.
- Eijkhout HW, Der Meer JW, Kallenberg CG, et al. The effect of two different dosages of intravenous immunoglobulin on the incidence of recurrent infections in patients with primary hypogammaglobulinemia. A randomized, double-blind, multicenter crossover trial. Ann Intern Med. 2001;135:165-174.
- Rofman CM, Gelfand EW. Replacement therapy with high dose intravenous gamma-globulin improves chronic sinopulmonary disease in patients with hypogammaglobulinemia. Pediatr Infect Dis J. 1988;7:S92-S96.
- Ballow M, Berger M, Bonilla FA, et al. Pharmacokinetics and tolerability of a new intravenous immunoglobulin preparation, IGIV-C, 10% (Gamunex, 10%). Vox Sang. 2003;84:202-210.
- Ochs HD, Pinciaro PJ, The Octagam Study Group. Octagam(R) 5%, an intravenous IgG product, is efficacious and well tolerated in subjects with primary immunodeficiency diseases. J Clin Immunol. 2004;24:309-314.
- Cayco AV, Perazella MA, Hayslett JP. Renal insufficiency after intravenous immune globulin therapy: a report of two cases and an analysis of the literature. J Am Soc Nephrol. 1997;8:1788-1794.
- Copelan EA, Strohm PL, Kennedy MS, Tutschka PJ. Hemolysis following intravenous immune globulin therapy. Transfusion. 1986;26:410-412.
- Wilson JR, Bhoopalam H, Fisher M. Hemolytic anemia associated with intravenous immunoglobulin. Muscle Nerve. 1997;20:1142-1145.
- Thomas MJ, Misbah SA, Chapel HM, Jones M, Elrington G, Newsom-Davis J. Hemolysis after high-dose intravenous Ig. Blood. 1993;82:3789.
- Kessary-Shoham H, Levy Y, Shoenfeld Y, Lorber M, Gershon H. In vivo administration of intravenous immunoglobulin (IVIG) can lead to enhanced erythrocyte sequestration. J Autoimmun. 1999;13:129-135.
- Rizk A, Gorson KC, Kenney L, Weinstein R. Transfusion-related acute lung injury after the infusion of MG. Transfusion. 2001;41:264-268.
- Brannagan TH, III, Nagle KJ, Lange DJ, Rowland LP. Complications of intravenous immune globulin treatment in neurologic disease. Neurology. 1996;47:674-677.
- Dalakas MC. High-dose intravenous immunoglobulin and serum viscosity: risk of precipitating thromboembolic events. Neurology. 1994;44:223-226.
- Elkayam O, Paran D, Milo R, et al. Acute myocardial infarction associated with high dose intravenous immunoglobulin infusion for autoimmune disorders. A study of four cases. Ann Rheum Dis. 2000;59:77-80.
- Gomperts ED, Darr F. Rapid infusion of intravenous immunoglobulin in patients with neuromuscular diseases. Neurology. 2002;58:1444.
- Haplea SS, Farrar JT, Gibson GA, Laskin M, Pizzi LT, Ashbury AK. Thromboembolic events associated with intravenous immunoglobulin therapy [abstract]. Neurology. 1997;48:A54.
- Harkness K, Howell SJ, Davies-Jones GA. Encephalopathy associated with intravenous immunoglobulin treatment for Guillain-Barre syndrome. J Neurol Neurosurg Psychiatry. 1996;60:586.
- Kwan T, Keith P. Stroke following intravenous immunoglobulin infusion in a 28-year-old male with common variable immune deficiency: a case report and literature review. Can J Allergy Clin Immunol. 1999;4:250-253.
- Wolberg AS, Kon RH, Monroe DM, Hoffman M. Coagulation factor XI is a contaminant in intravenous immunoglobulin preparations. Am J Hematol. 2000;65:30-34.
- Woodruff RK, Grigg AP, Firkin FC, Smith IL. Fatal thrombotic events during treatment of autoimmune thrombocytopenia with intravenous immunoglobulin in elderly patients. Lancet. 1986;2:217-218.
- Hammarstrom L, Smith CI. Placental transfer of intravenous immunoglobulin. Lancet. 1986;1:681.
- Morell A, Sidiropoulos D, Herrmann U, et al. IgG subclasses and antibodies to group B streptococci, pneumococci, and tetanus toxoid in preterm neonates after intravenous infusion of immunoglobulin to the mothers. Pediatr Res. 1986;20:933-936.
- Sidiropoulos D, Herrmann U, Jr., Morell A, von Murali G, Barandun S. Transplacental passage of intravenous immunoglobulin in the last trimester of pregnancy. J Pediatr. 1986;109:505-508.
- Pierce LR, Jain N. Risks associated with the use of intravenous immunoglobulin. Transfusion Med Rev. 2003;17:241-251.
- Tan E, Hajinazarian M, Bay W, Neff J, Mendell JR. Acute renal failure resulting from intravenous immunoglobulin therapy. Arch Neurol. 1993;50:137-139.
- Morbidity and Mortality Weekly Report. Measles, Mumps, and Rubella; Vaccine use and strategies for elimination of measles, rubella, and congenital rubella syndrome and control of mumps. Recommendations for the Advisory Committee on Immunization Practices (ACIP). 98 A.D.47.
- Peter G. Summary of major changes in the 1994 Red Book: American Academy of Pediatrics. Report of the Committee on Infectious Disease. Pediatrics. 1994;93:1000-1002.
- Siber GR, Werner BG, Halsey NA, et al. Interference of immune globulin with measles and rubella immunization. J Pediatr. 1993;122:204-211.
- Data on file, Baxter Healthcare Corporation.

**Baxter**





# Aralast

## [alpha<sub>1</sub>-proteinase inhibitor (human)]

### Solvent Detergent Treated Nanofiltered

#### DESCRIPTION

ARALAST is a sterile, stable, lyophilized preparation of purified human alpha<sub>1</sub>-proteinase inhibitor (α<sub>1</sub>-PI), also known as alpha<sub>1</sub>-antitrypsin.<sup>1</sup>

ARALAST is prepared from large pools of human plasma by using the Cohn-Oncley cold alcohol fractionation process, followed by purification steps including polyethylene glycol and zinc chloride precipitations and ion exchange chromatography. To reduce the risk of viral transmission, the manufacturing process includes treatment with a solvent detergent (SD) mixture [tri-n-butyl phosphate and polysorbate 80] to inactivate enveloped viral agents such as HIV and Hepatitis B and C. In addition, a nanofiltration step is incorporated prior to final sterile filtration to reduce the risk of transmission of non-enveloped viral agents. Based on *in vitro* studies, the process used to produce ARALAST has been shown to inactivate and/or partition various viruses as shown in the table below.<sup>2</sup>

Processing Step	Elimination of Deliberately Added Virus (Number of logs inactivated/removed)				
	HIV-1*	BVD†	PRV‡	HAV‡	PPV‡
Alcohol Fractionation	≥ 4.8	N/A	N/A	N/A	N/A
Solvent Detergent Treatment	≥ 7.2	≥ 4.8	≥ 5.1	N/A	N/A
Nanofiltration	N/A	≥ 6.0	≥ 5.5	8.6	≥ 5.8
Accumulated Reduction	≥ 12.0	≥ 10.8	≥ 10.6	8.6	≥ 5.8

\* HIV-1: Fractionation units log<sub>10</sub> SFU SD Treatment units log<sub>10</sub> TCID<sub>50</sub>/mL

† BVD (Bovine Viral Diarrhea, model for Hepatitis C Virus and other lipid enveloped RNA viruses); PRV (Pseudorabies Virus, model for large lipid enveloped DNA viruses); SD Treatment units log<sub>10</sub> PFU/mL, Nanofiltration units log<sub>10</sub> PFU

‡ HAV (Hepatitis A), PPV (Porcine Parvovirus) Nanofiltration units log<sub>10</sub> PFU

N/A Not Applicable

The unconstituted, lyophilized cake should be white or off-white to slightly yellow-green or yellow in color. When reconstituted as directed, the concentration of α<sub>1</sub>-PI is not less than 16 mg/mL and the specific activity is not less than 0.55 mg active α<sub>1</sub>-PI/mg total protein. The composition of the reconstituted product is as follows:

Component	Quantity/mL
Elastase Inhibitory Activity	NLT 400 mg Active α <sub>1</sub> -PI/0.5 g vial *
	NLT 800 mg Active α <sub>1</sub> -PI/1.0 g vial **
Albumin	NMT 5 mg/mL
Polyethylene Glycol	NMT 112 µg/mL
Polysorbate 80	NMT 50 µg/mL
Sodium	NMT 230 mEq/L
Tri-n-butyl Phosphate	NMT 1.0 µg/mL
Zinc	NMT 3 ppm

NLT: Not Less Than

NMT: Not More Than

\* Reconstitution volume: 25 mL/0.5 g vial

\*\* Reconstitution volume: 50 mL/1.0 g vial

Each vial of ARALAST is labeled with the amount of functionally active α<sub>1</sub>-PI expressed in mg/vial. The formulation contains no preservative. The pH of the solution ranges from 7.2 to 7.8. Product must only be administered intravenously.

#### CLINICAL PHARMACOLOGY

ARALAST functions in the lungs to inhibit serine proteases such as neutrophil elastase (NE), which is capable of degrading protein components of the alveolar walls and which is chronically present in the lung. In the normal lung, α<sub>1</sub>-PI is thought to provide more than 90% of the anti-NE protection in the lower respiratory tract.<sup>3,4</sup>

α<sub>1</sub>-PI deficiency is an autosomal, co-dominant, hereditary disorder characterized by low serum and lung levels of α<sub>1</sub>-PI.<sup>1,3,5</sup> Severe forms of the deficiency are frequently associated with slowly progressive, moderate-to-severe panacinar emphysema that most often manifests in the third to fourth decades of life, resulting in a significantly lower life expectancy.<sup>1,3,4,6</sup> Individuals with α<sub>1</sub>-PI deficiency have little protection against NE released by a chronic, low-level of neutrophils in their lower respiratory tract, resulting in a protease:protease inhibitor imbalance in the lung.<sup>7</sup> The emphysema associated with α<sub>1</sub>-PI deficiency is typically worse in the lower lung zones.<sup>8</sup> It is believed to develop because there are insufficient amounts of α<sub>1</sub>-PI in the lower respiratory tract to inhibit NE. This imbalance allows unopposed destruction of the connective tissue framework of the lung parenchyma.<sup>7,8</sup>

There are a large number of phenotypic variants of this disorder.<sup>1,3,4</sup> Individuals with the PiZZ variant typically have serum α<sub>1</sub>-PI levels less than 35% of the average normal level.<sup>1,3</sup> Individuals with the Pi(null)(null) variant have undetectable α<sub>1</sub>-PI protein in their serum.<sup>1,3</sup> Individuals with these low serum α<sub>1</sub>-PI levels, i.e., less than 11 µmolar (80 mg/dL), have an unknown risk of developing emphysema over their lifetimes. Two Registry studies have shown risks of 54.2 and 57.0%.<sup>1,3,9</sup> The risk of accelerated development and progression of emphysema in individuals with severe α<sub>1</sub>-PI deficiency is higher in smokers than in ex-smokers or non-smokers.<sup>3</sup> The deficiency in α<sub>1</sub>-PI represents one of the most common, potentially lethal hereditary disorders.<sup>4</sup>

A clinical study was conducted to compare ARALAST (test drug) to a commercially available preparation of α<sub>1</sub>-PI (Prolastin®), manufactured by Bayer Corporation. All subjects were to have been diagnosed as having congenital α<sub>1</sub>-PI deficiency and emphysema but no α<sub>1</sub>-PI augmentation therapy within the preceding six months.

Twenty-eight subjects were randomized to receive either test drug or control drug, 60 mg/kg intravenously per week, for 10 consecutive weeks. Two subjects withdrew from the study prematurely: 1 subject receiving ARALAST withdrew consent after 6 infusions; 1 subject receiving Prolastin® withdrew after 1 infusion due to pneumonia following unscheduled bronchoscopy to remove a foreign body. Trough levels of serum α<sub>1</sub>-PI (antigenic determination) and anti-NE capacity (functional determination) were measured prior to treatment at Weeks 8 through 11. Following their first 10 weekly infusions, the subjects who were receiving control drug were switched to ARALAST while those who already were receiving ARALAST continued to receive it. Maintenance of mean serum α<sub>1</sub>-PI trough levels was assessed prior to treatments at Weeks 12 through 24. Bronchoalveolar lavages (BALs) were performed on subjects at baseline and prior to treatment at Week 7. The epithelial lining fluid (ELF) from each BAL meeting acceptance criteria was analyzed for the α<sub>1</sub>-PI level and anti-NE capacity.

With weekly augmentation therapy, a gradual increase in peak and trough serum α<sub>1</sub>-PI levels was noted, with stabilization after several weeks. The metabolic half-life of ARALAST was 5.9 days. Serum anti-NE capacity trough levels rose substantially in all subjects by Week 2, and by Week 3, serum anti-NE capacity trough levels exceeded 11 µM in the majority of subjects. With few exceptions, levels remained above this recommended threshold level in individual subjects for the duration of the period Weeks 3 through 24 on study. Although only five of fourteen subjects (35.7%) receiving ARALAST had BALs meeting acceptance criteria for analysis at both baseline and Week 7, a statistically significant increase in the antigenic level of α<sub>1</sub>-PI in the ELF was observed. No statistically significant increase in the anti-NE capacity in the ELF was detected.

Viral serology of all subjects was determined periodically throughout the study, including testing for antibodies to hepatitis A (HAV) and C (HCV), presence of circulating HBsAg, and presence of antibodies to HIV-1, HIV-2, and Parvovirus B-19. Subjects who were seronegative to parvovirus B-19 at enrollment were retested by PCR at Week 2. There were no seroconversions in subjects treated with ARALAST through Week 24. None of the subjects became HBsAg positive during the study, although five of 13 (38%) evaluable subjects in the test group and eight of 13 (62%) in the control group had not been vaccinated to hepatitis B. No patient developed antibodies against α<sub>1</sub>-PI.

It was concluded that at a dose of 60 mg/kg administered intravenously once weekly, ARALAST and the control α<sub>1</sub>-PI preparation had similar effects in maintaining target serum α<sub>1</sub>-PI trough levels and increasing antigenic levels of α<sub>1</sub>-PI in epithelial lining fluid (ELF) with maintenance augmentation therapy.

#### INDICATIONS AND USAGE

##### Congenital Alpha<sub>1</sub>-Proteinase Inhibitor Deficiency

ARALAST is indicated for chronic augmentation therapy in patients having congenital deficiency of α<sub>1</sub>-PI with clinically evident emphysema. Clinical and biochemical studies have demonstrated that with such therapy, ARALAST is effective in maintaining target serum α<sub>1</sub>-PI trough levels and increasing α<sub>1</sub>-PI levels in epithelial lining fluid (ELF). Clinical data demonstrating the long-term effects of chronic augmentation or replacement therapy of individuals with ARALAST are not available.

Safety and effectiveness in pediatric patients have not been established.

ARALAST is not indicated as therapy for lung disease patients in whom congenital α<sub>1</sub>-PI deficiency has not been established.

#### CONTRAINDICATIONS

ARALAST is contraindicated in individuals with selective IgA deficiencies (IgA level less than 15 mg/dL) who have known antibody against IgA, since they may experience severe reactions, including anaphylaxis, to IgA which may be present.

#### WARNINGS

**Because ARALAST is derived from pooled human plasma, it may carry a risk of transmitting infectious agents, e.g., viruses and theoretically, the Creutzfeldt-Jakob disease (CJD) agent.** Stringent procedures designed to reduce the risk of adventitious agent transmission have been employed in the manufacture of this product, from the screening of plasma donors and the collection and testing of plasma through the application of viral elimination/reduction steps such as alcohol fractionation, PEG precipitation, solvent detergent treatment, and nanofiltration. Despite these measures, such products can still potentially transmit disease; therefore, the risk of infectious agents cannot be totally eliminated. ALL infections thought by a physician possibly to have been transmitted by this product should be reported to the manufacturer at 1-800-423-2090 (US). The physician should weigh the risks and benefits of the use of this product and should discuss these with the patient.

The rate of administration specified in DOSAGE AND ADMINISTRATION should be closely followed, at least until the physician has had sufficient experience with a given patient. Vital signs should be monitored continuously and the patient should be carefully observed throughout the infusion. **IF ANAPHYLACTIC OR SEVERE ANAPHYLACTOID REACTIONS OCCUR, THE INFUSION SHOULD BE DISCONTINUED IMMEDIATELY.** Epinephrine and other appropriate supportive therapy should be available for the treatment of any acute anaphylactic or anaphylactoid reaction.

**Baxter**

## PRECAUTIONS

### General

ARALAST should be administered within three (3) hours after the reconstituted product is warmed to room temperature. Partially used vials should be discarded and not saved for future use. The solution contains no preservative.

ARALAST should be administered alone, without mixing with other agents or diluting solutions.

### Pregnancy Category C

Animal reproduction studies have not been conducted with ARALAST. It is also not known whether ARALAST can cause fetal harm when administered to pregnant women or can affect reproductive capacity.

### Nursing Mothers

It is not known whether alpha<sub>1</sub>-proteinase inhibitor is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when ARALAST is administered to a nursing woman.

### Pediatric Use

Safety and effectiveness in pediatric patients have not been established.

## ADVERSE REACTIONS

ARALAST was evaluated for up to 96 weeks in 27 subjects with a congenital deficiency of  $\alpha_1$ -PI and clinically evident emphysema. The number of subjects with an adverse event, regardless of causality, was 22 of 27 (81.5%). The number of subjects with an adverse event deemed possibly, probably, or definitely related to study drug was 7 of 27 (25.9%).

The frequency of infusions associated with an adverse event, regardless of causality, was 108 of 1127 (9.6%) infusions administered per protocol. The most common symptoms were pharyngitis (1.6%), headache (0.7%), and increased cough (0.6%). Symptoms of bronchitis, sinusitis, pain, rash, back pain, viral infection, peripheral edema, bloating, dizziness, somnolence, asthma, and rhinitis were each associated with  $\geq 0.2\%$  of infusions. All symptoms were mild to moderate in severity.

The overall frequency of adverse events deemed to be possibly, probably, or definitely related to study drug was 15 of 1127 (1.3%) infusions. The most common symptoms included headache (0.3%) and somnolence (0.3%). Symptoms of chills and fever, vasodilation, dizziness, pruritus, rash, abnormal vision, chest pain, increased cough, and dyspnea were each associated with one (0.1%) infusion. Five (5) of 27 (18.5%) subjects experienced eight (8) serious adverse reactions during the study. None of these were considered to be causally related to the administration of ARALAST.

Twenty-six (26) of 27 (96.3%) subjects experienced a total of 94 upper and lower respiratory-tract infections during the 96-week study (median: 3.0; range: 1 to 8; mean  $\pm$  SD:  $3.6 \pm 2.3$  infections). Twenty-eight (29.8%) of the respiratory infections occurred in 19 (70.4%) subjects during the first 24 weeks of the 96-week study suggesting that the risk of infection did not change with time on ARALAST. In a post-hoc analysis, subjects experienced a range of 0 to 8 exacerbations of COPD over the 96-week study with a median of less than one exacerbation per year (median: 0.61; mean  $\pm$  SD:  $0.83 \pm 0.87$  exacerbations per year).

Treatment-emergent elevations ( $>$  two times the upper limit of normal) in aminotransferases (ALT or AST), up to 3.7 times the upper limit of normal, were noted in 3 of 27 (11.1%) subjects. Elevations were transient lasting three months or less. No subject developed any evidence of viral hepatitis or hepatitis seroconversion while being treated with ARALAST, including 13 evaluable subjects who were not vaccinated against hepatitis B.

No clinically relevant alterations in blood pressure, heart rate, respiratory rate, or body temperature occurred during infusion of ARALAST. Mean hematology and laboratory parameters were little changed over the duration of the study, with individual variations not clinically meaningful.

During the initial 10 weeks of the study, subjects were randomized to receive either ARALAST or a commercially available preparation of  $\alpha_1$ -PI (Prolastin®). The overall frequency, severity and symptomatology of adverse reactions were similar in both the ARALAST and control drug groups. There were two serious adverse events in the control group, both of which were considered to be possibly related to the control drug. These included chest pain, dyspnea and bilateral pulmonary infiltrates in one individual that withdrew from the study prematurely following an unscheduled bronchoscopy to remove a foreign body and the other, a positive seroconversion to Parvovirus B-19. There were no serious adverse events or seroconversions reported for the ARALAST group during the 96 week study period. No subject developed an antibody to  $\alpha_1$ -PI.

## DOSE AND ADMINISTRATION

### Chronic Augmentation Therapy

FOR INTRAVENOUS USE ONLY. The recommended dosage of ARALAST is 60 mg/kg body weight administered once weekly by intravenous infusion. Each vial of ARALAST has the functional activity, as determined by inhibition of porcine pancreatic elastase, stated on the label. Administration of ARALAST within three hours after reconstitution is recommended to avoid the potential ill effect of any inadvertent microbial contamination occurring during reconstitution. Discard any unused contents.

### Infusion Rate

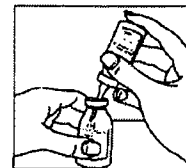
ARALAST should be administered at a rate not exceeding 0.08 mL/kg body weight/minute. If adverse events occur, the rate should be reduced or the infusion interrupted until the symptoms subside. The infusion may then be resumed at a rate tolerated by the subject.

## RECONSTITUTION

### Use Aseptic Technique

1. ARALAST and diluent should be at room temperature before reconstitution.
2. Remove caps from the diluent and product vials.

3. Swab the exposed stopper surfaces with alcohol.
4. Remove cover from one end of the double-ended transfer needle. Insert the exposed end of the needle through the center of the stopper in the DILUENT vial.
5. Remove plastic cap from the other end of the double-ended transfer needle now seated in the stopper of the diluent vial. To reduce any foaming, invert the vial of diluent and insert the exposed end of the needle through the center of the stopper in the PRODUCT vial at an angle, making certain that the diluent vial is always above the product vial. The angle of insertion directs the flow of diluent against the side of the product vial. Refer to Figure below. The vacuum in the vial is sufficient to allow transfer of all of the diluent.
6. Disconnect the two vials by removing the transfer needle from the diluent vial stopper. Remove the double-ended transfer needle from the product vial and discard the needle into the appropriate safety container.
7. Let the vial stand until most of the contents is in solution, then GENTLY swirl until the powder is completely dissolved. Reconstitution requires no more than five minutes for a 0.5 gram vial and no more than 10 minutes for a 1.0 gram vial.
8. DO NOT SHAKE THE CONTENTS OF THE VIAL. DO NOT INVERT THE VIAL UNTIL READY TO WITHDRAW CONTENTS.
9. Use within three hours of reconstitution.
10. Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration. The reconstituted product should be a colorless or slightly yellow to yellowish-green solution. When reconstitution procedure is strictly followed, a few small visible particles may occasionally remain. These will be removed by the microaggregate filter.
11. Reconstituted product from several vials may be pooled into an empty, sterile IV solution container by using aseptic technique. A sterile 20 micron filter is provided for this purpose.



## HOW SUPPLIED

ARALAST is supplied as a sterile, nonpyrogenic, lyophilized powder in single-dose vials. The following product packages are available: 0.5 g (NDC 0944-2801-01) and 1.0 g (NDC 0944-2801-02). A suitable volume of Sterile Water for Injection, USP diluent is provided (25 mL/0.5 g vial; 50 mL/1.0 g vial). Each vial is labeled with the total  $\alpha_1$ -PI functional activity in mg. ARALAST is packaged with a sterile double-ended transfer needle and a sterile 20 micron filter.

## STORAGE

ARALAST should be stored at 2° to 8°C (35° to 46°F). ARALAST may be removed from refrigeration and stored at temperatures not to exceed 25°C (77°F). Product removed from refrigeration must be used within one month. Do not freeze. Do not use after the expiration date printed on the label.

## Rx only

## REFERENCES

1. Brantly M, Nukiwa J, Crystal RG. Molecular basis of alpha-1-antitrypsin deficiency. Am J Med 1988 (Suppl 6A):84:13-31.
2. Data on file at Baxter Healthcare Corporation.
3. Crystal RG, Brantly ML, Hubbard RC, Curiel DT, et al. The alpha-1-antitrypsin gene and its mutations: Clinical consequences and strategies for therapy. Chest 1989;95:196-208.
4. Crystal RG.  $\alpha_1$ -Antitrypsin deficiency: pathogenesis and treatment. Hospital Practice 1991;Feb.15:81-94.
5. Hutchison DCS. Natural history of alpha-1-protease inhibitor deficiency. Am J Med 1988;84(Suppl 6A):3-12.
6. Hubbard RC, Crystal RG. Alpha-1-antitrypsin augmentation therapy for alpha-1-antitrypsin deficiency. Am J Med 1988;84(Suppl 6A):52-62.
7. Ogushi F, Fells GA, Hubbard RC, et al. Z-Type  $\alpha_1$ -antitrypsin as an inhibitor of neutrophil elastase. J Clin Investigation 1987;80:1366-1374.
8. Buist SA, Burrows B, Cohen A, et al. Guidelines for the approach to the patient with severe hereditary alpha-1-antitrypsin deficiency. Am Rev Respir Dis 1989;140:1494-1497.
9. Stoller JK, Brantly M, Fleming LE, et al. Formation and current results of a patient-organized registry for  $\alpha_1$ -antitrypsin deficiency. Chest 2000; 118(3):843-848.

Baxter and ARALAST are registered trademarks of Baxter International Inc.

U.S. Patent No.: 5,616,693

U.S. Patent No.: 5,981,715

Other U.S. Patents Pending

DATE OF REVISION: January 2007

**Baxter Healthcare Corporation**

Westlake Village, CA 91362

U.S. License No. 140

0707303

**Baxter**

**ANTIHEMOPHILIC FACTOR (HUMAN)****ALPHANATE®****Solvent Detergent / Heat Treated****DESCRIPTION**

Antihemophilic Factor (Human), Alphanate®, Solvent Detergent / Heat Treated, is a single dose, sterile, lyophilized concentrate of Factor VIII (AHF) intended for intravenous administration in the treatment of hemophilia A, or acquired Factor VIII deficiency.

Alphanate® is prepared from pooled human plasma by cryoprecipitation of the Factor VIII, fractional solubilization, and further purification employing heparin-coupled, cross-linked agarose which has an affinity to the heparin binding domain of vWt/FVIII:C complex.<sup>1</sup> The product is treated with a mixture of tri(n-butyl) phosphate (TNBP) and polysorbate 80 to reduce the risks of transmission of viral infection. In order to provide an additional safeguard against potential non-lipid enveloped viral contaminants, the product is also subjected to a 80 °C heat treatment step for 72 hours. However, no procedure has been shown to be totally effective in removing viral infectivity from coagulation factor products.

Alphanate® is labeled with the antihemophilic factor potency (Factor VIII:C activity) expressed in International Units (IU) per vial, which is referenced to the WHO International Standard.

Alphanate® contains Albumin (Human) as a stabilizer, resulting in a final container concentrate with a specific activity of at least 5 IU FVIII:C/mg total protein. Prior to the addition of the Albumin (Human) stabilizer, the specific activity is significantly higher.

When reconstituted with the appropriate volume of Sterile Water for Injection, USP, Alphanate® contains 0.3 - 0.9 g Albumin (Human)/100 mL; NMT 5 mmol calcium/L; NMT 750 µg glycine/IU FVIII:C; NMT 1.0 U heparin/mL; 10 - 40 mmol histidine/L; NMT 0.1 mg imidazole/mL; 50 - 200 mmol arginine/L; NMT 1.0 µg polyethylene glycol and polysorbate 80/IU FVIII:C; NMT 10 mEq sodium/vial; and NMT 0.1 µg TNBP/IU FVIII:C.

**CLINICAL PHARMACOLOGY**

Antihemophilic Factor (Human) is a constituent of normal plasma and is required for clotting. The administration of Alphanate® temporarily increases the plasma level of this clotting factor, thus minimizing the hazard of hemorrhage.<sup>2,3</sup> Following the administration of Alphanate® during clinical trials, the mean *in vivo* half-life of Factor VIII observed in 12 adult subjects with severe hemophilia A was 17.9 ± 9.6 hours. In this same study, the *in vivo* recovery was 96.7 ± 14.5% at 10 minutes postinfusion.<sup>4</sup> Recovery at 10 minutes postinfusion was also determined as 2.4 ± 0.4 IU FVIII rise/dL plasma per IU FVIII infused/kg body weight.<sup>4</sup>

The solvent detergent treatment process has been shown by Horowitz, et al., to provide a high level of virus kill without compromising protein structure and function.<sup>5</sup> The susceptibility of human pathogenic viruses such as the human immunodeficiency viruses, hepatitis viruses, as well as marker viruses such as sindbis virus and vesicular stomatitis virus (VSV), to inactivation by organic solvent detergent treatment has been discussed in the literature.<sup>6</sup>

*In vitro* inactivation studies to evaluate the solvent detergent treatment step used in the manufacture of Alphanate® employed an assay with a sensitivity of 2 logs of virus for the marker viruses, vesicular stomatitis virus (VSV) and sindbis virus. The studies demonstrated a log kill of ≥4.1 for VSV and ≥4.7 for sindbis virus. Greater than or equal to 11.1 logs of HIV-1 and greater than or equal to 6.1 logs of HIV-2 were inactivated by the solvent detergent treatment step. The number of viral particles inactivated by the process represents the maximum amount of virus added initially to the sample, thus the results of the study indicate that all the added HIV virus was killed.<sup>4</sup>

In another study, the dry heat cycle of 80 °C for 72 hours of the Alphanate® manufacturing process was shown to inactivate greater than or equal to 5.8 logs of hepatitis A virus (HAV).

In a different study, the following steps in the manufacturing process of Alphanate® were evaluated for virus reduction/removal capability: precipitation with 3.5% polyethylene glycol (PEG), solvent detergent treatment with 0.3% tri-n-butyl phosphate and 1.0% polysorbate 80, heparin-actigel-ALD chromatography, lyophilization of Factor VIII and heat treatment at 80 °C for 72 hours. The following viruses were used in these studies: bovine herpes (BHV), bovine viral diarrhea virus (BVD), human poliovirus Sabin type 2 (POL), canine parvovirus (CPV) and human immunodeficiency virus, type 1 (HIV-1).

Table 1 summarizes the reduction factors for each virus evaluated for each viral inactivation/removal step validated in the manufacturing process of Alphanate®.<sup>4</sup>

However, no treatment method has yet been shown capable of totally eliminating all potential infective virus in preparations of coagulation factor concentrates.

**Table 1**

Virus Reduction (log <sub>e</sub> )	Processing Step					
	3.5% PEG Precipitation	Solvent Detergent treatment	Column chromatography	Lyophilization of Factor VIII	Dry heat Cycle (80 °C, 72h)	Total Log Removal
BHV	< 1.0	≥ 8.0	7.6	1.3	2.1	≥ 19.0
BVD	< 1.0	≥ 4.5	< 1.0	< 1.0	≥ 4.9	≥ 9.4
POL	3.3	—	< 1.0	3.4	≥ 2.5	≥ 9.2
CPV	1.2	—	< 1.0	< 1.0	4.1	5.3
VSV	—	≥ 4.1	—	—	—	≥ 4.1
Sindbis	—	≥ 4.7	—	—	—	≥ 4.7
HIV-1	< 1.0	≥ 11.1	≥ 2.0	—	—	≥ 13.1
HIV-2	—	≥ 6.1	—	—	—	≥ 6.1
HAV	—	—	—	2.1	≥ 5.8	≥ 7.9

**INDICATIONS AND USAGE**

Antihemophilic Factor (Human), Alphanate®, is indicated for the prevention and control of bleeding in patients with Factor VIII deficiency due to hemophilia A or acquired Factor VIII deficiency.<sup>7</sup> No clinical trials have as yet been conducted using Alphanate® for treatment of von Willebrand's disease, therefore the product is not approved for this use.

**CONTRAINDICATIONS**

None known.

**WARNINGS**

Because Antihemophilic Factor (Human), Alphanate® is made from pooled human plasma, it may carry a risk of transmitting infectious agents, e.g., viruses, and theoretically, the Creutzfeldt-Jakob disease (CJD) agent. Stringent procedures designed to reduce the risk of adventitious agent transmission have been employed in the manufacture of this product, from the screening of plasma donors and the collection and testing of plasma, through the application of viral elimination/reduction steps such as solvent detergent and heat treatment in the manufacturing process. Despite these measures, such products can still potentially transmit disease; therefore, the risk of infectious agents cannot be totally eliminated. All infections thought by a physician possibly to have been transmitted by this product should be reported to the manufacturer at 1-888-675-2762 (US) or 1-323-225-9735 (International). The physician should weigh the risks and benefits of the use of this product and should discuss these with the patient.

Individuals who receive infusions of blood or plasma products may develop signs and/or symptoms of some viral infections, particularly hepatitis C.<sup>8,9</sup> Incubation in a solvent detergent mixture during the manufacturing process is designed to reduce the risk of transmitting viral infection.<sup>8,9</sup> However, scientific opinion encourages hepatitis A and hepatitis B vaccinations for patients with hemophilia at birth or at the time of diagnosis.

**PRECAUTIONS****General**

Antihemophilic Factor (Human), Alphanate®, should not be administered at a rate exceeding 10 mL/minute. Rapid administration of a Factor VIII concentrate may result in vasomotor reactions.

Some patients develop inhibitors to Factor VIII. Factor VIII inhibitors are circulating antibodies (i.e., globulins) that neutralize the procoagulant activity of Factor VIII. No studies have been conducted with Alphanate® to evaluate inhibitor formation. Therefore, it is not known whether there are greater, lesser or the same risks of developing inhibitors due to the use of this product than there are with other antihemophilic factor preparations. Patients with these inhibitors may not respond to treatment with Antihemophilic Factor (Human), or the response may be much less than would otherwise be expected; therefore, larger doses of Antihemophilic Factor (Human) are often required. The management of bleeding in patients with inhibitors requires careful monitoring, especially if surgical procedures are indicated.<sup>10-12</sup>

Nursing personnel, and others who administer this material, should exercise appropriate caution when handling due to the risk of exposure to viral infection. Discard any unused contents into the appropriate safety container. Discard administration equipment after single use into the appropriate safety container. Do not resterilize components.

**Information for Patients**

Patients should be informed of the early symptoms and signs of hypersensitivity reaction, including hives, generalized urticaria, chest tightness, dyspnea, wheezing, faintness, hypotension, and anaphylaxis. Patients should be advised to discontinue use of the product and contact their physician and/or seek immediate emergency care, depending on the severity of the reaction, if these symptoms occur.

Some viruses, such as parvovirus B19 or hepatitis A, are particularly difficult to remove or inactivate at this time. Parvovirus B19 may most seriously affect seronegative pregnant women, or immunocompromised individuals. The majority of parvovirus B19 and hepatitis A infections are acquired by environmental (natural) sources.

**Pregnancy Category C**

Animal reproduction studies have not been conducted with Alphanate®. Therefore, it is not known whether it can cause fetal harm when administered to a pregnant woman or affect the reproductive capacity of a woman. Alphanate® should be given to a pregnant woman only if clearly needed.

**Pediatric Use**

Clinical trials for safety and effectiveness in pediatric patients 16 years of age and younger have not been conducted. Across well controlled half-life and recovery clinical trial in patients previously treated with Factor VIII concentrates for Hemophilia A, the one pediatric patient receiving Alphanate® (solvent detergent) responded similarly when compared with 12 adult patients.<sup>4</sup> No adverse events were reported in either pediatric or adult patients with Alphanate®.<sup>4</sup>

**ADVERSE REACTIONS**

Adverse reactions may include urticaria, fever, chills, nausea, vomiting, headache, somnolence, or lethargy.

Occasionally, mild reactions occur following the administration of Antihemophilic Factor (Human)<sup>13</sup>, such as allergic reactions, chills, nausea, or stinging at the infusion site. If a reaction is experienced, and the patient requires additional Antihemophilic Factor (Human), product from a different lot should be administered.

Massive doses of Antihemophilic Factor (Human) have rarely resulted in acute hemolytic anemia, increased bleeding tendency or hyperfibrinogenemia.<sup>14</sup> Alphanate® contains blood group specific isoagglutinins and, when large and/or frequent doses are required in patients of blood groups A, B, or AB, the patient should be monitored for signs of intravascular hemolysis and falling hematocrit. Should this condition occur, thus leading to progressive hemolytic anemia, the administration of serologically compatible type O red blood cells should be considered or the administration of Antihemophilic Factor (Human) produced from group-specific plasma should be considered.



## DOSAGE AND ADMINISTRATION

### For adult usage:

Following reconstitution with the supplied diluent, Alphanate® should be administered intravenously within three hours after reconstitution to avoid the potential ill effect of any inadvertent bacterial contamination occurring during reconstitution. Alphanate® may be administered by injection (plastic disposable syringes are recommended). Administer at room temperature, do not refrigerate after reconstitution, and discard any unused contents into the appropriate safety container.

Antihemophilic factor potency (Factor VIII:C activity) is expressed in International Units (IU) on the product label. One unit approximates the activity in one mL of normal human plasma. Replacement therapy studies have shown a linear dose-response relationship with a 2.0-2.5% increase in Factor VIII activity for each unit of Factor VIII:C per kg of body weight transfused, from which an approximate factor of 0.5 IU/kg can be calculated.<sup>15,16</sup>

The following formula provides a guide for dosage calculation (the plasma Factor VIII may vary depending upon the age, weight, severity of hemorrhage, or surgical procedure of the patient):

Body weight (in kg)	X	0.50 IU/kg	X	Factor VIII Increase Desired (Percent)	=	Number of Factor VIII:C IU Required
Example: 50 kg	X	0.50 IU/kg	X	30 (% increase)	=	750 IU Factor VIII:C

Mild to moderate hemorrhages can usually be treated with a single administration of Alphanate® sufficient to raise the plasma Factor VIII level to 20 to 30%. In the event of more serious hemorrhage, the patient's plasma Factor VIII level should be raised to 30 to 50%. Infusions are generally required at twice daily intervals over several days.<sup>16</sup>

Surgery in patients with Factor VIII deficiency requires that postoperatively the Factor VIII level be raised to 50 to 80% and maintained at or above 30% for approximately two weeks. For dental extractions, the Factor VIII level should be raised to 50% immediately prior to the procedure; additional Alphanate® may be given if bleeding recurs.<sup>17</sup>

In patients with severe Factor VIII deficiency who experience frequent hemorrhages, Antihemophilic Factor (Human), Alphanate®, may be administered prophylactically on a daily or every other day schedule to raise the Factor VIII level to approximately 15%.<sup>18</sup>

Factor VIII levels should be monitored periodically to evaluate individual patient response to the dosage regime.

### For pediatric usage: See PRECAUTIONS

## RECONSTITUTION

### Always Use Aseptic Technique

1. Warm diluent (Sterile Water for Injection, USP) and concentrate (Alphanate®) to at least room temperature (but not above 37 °C).
2. Remove plastic caps from the diluent and concentrate vials.
3. Swab the exposed stopper surfaces with a cleansing agent such as alcohol. Do not leave excess cleansing agent on the stoppers.
4. Remove cover from one end of the double-ended transfer needle. Insert the exposed end of the needle through the center of the stopper in the DILUENT vial.
5. Remove plastic cap from the other end of the double-ended transfer needle now seated in the stopper of the diluent vial. To reduce any foaming, invert the vial of diluent and insert the exposed end of the needle through the center of the stopper in the CONCENTRATE vial at an angle, making certain that the diluent vial is always above the concentrate vial. The angle of insertion directs the flow of diluent against the side of the concentrate vial. Refer to Figure 1. There should be enough vacuum in the vial to transfer all of the diluent.

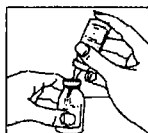


Figure 1

6. Disconnect the two vials by removing the transfer needle from the diluent vial stopper. Remove the double-ended transfer needle from the concentrate vial and discard the needle into the appropriate safety container.
7. Let the vial stand until contents are in solution, then GENTLY swirl until all concentrate is dissolved. Reconstitution requires less than 5 minutes.
8. DO NOT SHAKE THE CONTENTS OF THE VIAL. DO NOT INVERT THE CONCENTRATE VIAL UNTIL READY TO WITHDRAW CONTENTS.
9. Use as soon as possible after reconstitution.
10. After reconstitution, parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit. When reconstitution procedure is strictly followed, a few small particles may occasionally remain. The microaggregate filter will remove particles and the labeled potency will not be reduced.

## ADMINISTRATION BY SYRINGE

### Use Aseptic Technique

1. Peel cover from microaggregate filter package and securely install the syringe into the exposed Luer inlet of the filter, using a slight clockwise twisting motion.
2. Remove filter from packaging. Remove protective cover from the spike end of the filter.

3. Pull back plunger drawing sufficient air into the syringe to allow reconstituted product to be withdrawn as described in the next step.
4. Insert the spike end of the filter into the reconstituted concentrate vial. Inject air (Figure 2a) and withdraw the reconstituted product from the vial into the syringe (Figure 2b).

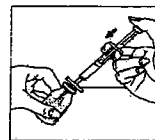


Figure 2a

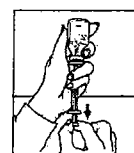


Figure 2b

5. Remove the filter from the syringe; discard the filter and the empty concentrate vial, into the appropriate safety container. Attach syringe to an infusion set, expel air from the syringe and infusion set. Perform venipuncture and administer slowly at a rate not exceeding 10 mL/minute.
6. If the patient is to receive more than one vial of concentrate, the infusion set will allow administration of multiple vials to be performed with a single venipuncture.
7. Discard all administration equipment after use into the appropriate safety container. Do not reuse.

## HOW SUPPLIED

Alphanate® is supplied in sterile, lyophilized form in single dose vials accompanied by a suitable volume of diluent (Sterile Water for Injection, USP), according to AHF potency. Each vial is labeled with the Factor VIII:C potency expressed in AHF International Units. Alphanate® is packaged with a double-ended transfer needle and microaggregate filter for use in administration.

## STORAGE

Alphanate® should be stored at temperatures between 2 and 8 °C. Do not freeze to prevent damage to diluent vial. May be stored at room temperature not to exceed 30 °C for up to 2 months. When removed from refrigeration, record the date removed on the space provided on the carton.

### Rx only

## REFERENCES

1. Fujimura, Y., Titani, K., Holland, L.Z., Roberts, J.R., Kostel, P., Ruggeri, Z.M., Zimmerman, T.S. A Heparin-Binding Domain of Human Von Willebrand Factor: Characterization and Localization to a Tryptic Fragment Extending from Amino Acid Residue Val-449 to Lys-728. *J Biol Chem* 1987, 262(4): 1734-1739.
2. Hershgold, E.J. Properties of Factor VIII (Antihemophilic Factor). In: Spaet, T.H., ed. *Progress in Hemostasis and Thrombosis*, Grune and Stratton Publisher, 1974, 2:99-139.
3. Ashenhurst, J.B., Langehenning, P.L., Seeler, R.A. Early Treatment of Bleeding Episodes with 10 U/Kg of Factor VIII. *Blood* 1977, 50:181.
4. Data on file at Grifols Biologicals Inc.
5. Edwards, C.A., Piet, M.P.J., Chin, S., Horowitz, B. Tri(n-Butyl) Phosphate/Detergent Treatment of Licensed Therapeutic and Experimental Blood Derivatives. *Vox Sang* 1987, 52:53-59.
6. Horowitz, B. Investigations into the Application of Tri(n-Butyl) Phosphate/Detergent Mixture to Blood Derivatives. In: Morgenthaler, J.J. ed. *Viral Inactivation in Plasma Products*, Karger, 1989, 56:83-96.
7. Eyster, M.E. Hemophilia: A Guide for the Primary Care Physician. *Postgraduate Medicine* 1978, 64:75-81.
8. Biggs, R. Jaundice and Antibodies Directed Against Factors VIII and IX in Patients Treated for Haemophilia or Christmas Disease in the United Kingdom. *Br J Haematol* 1974, 26:313-329.
9. Kasper, C.K., Kipnis, S.A. Hepatitis and Clotting Factor Concentrates. *JAMA* 1972, 221:510.
10. Kasper, C.K. Incidence and Course of Inhibitors Among Patients with Classic Hemophilia. *Thromb Diath Haemorrh* (Stuttg) 1973, 30:263-271.
11. Rizza, C.R., Biggs, R. The Treatment of Patients Who Have Factor VIII Antibodies. *Br J Haematol* 1973, 24:65-82.
12. Roberts, H.R., Knowles, M.R., Jones, T.L., McMillan, C. The Use of Factor VIII in the Management of Patients with Factor VIII Inhibitors. In: Brinkhous, K.M., ed. *Hemophilia and New Hemorrhagic States*, International Symposium, New York, University of North Carolina Press, 1970, pp.152-163.
13. Rizza, C.R., Biggs, R. Blood Products in the Management of Haemophilia and Christmas Disease. In: Poller, L., ed. *Recent Advances in Blood Coagulation*, Boston: Little Brown, 1969, pp.179-195.
14. Hathaway, W.E., Mahasandana, C., Clarke, S. Alteration of Platelet Function After Transfusion in Hemophilia. *Proc 14th Ann Mtg. Am Soc Hematol* 1971, Abstracts, 58, No. 88.
15. Shanbrom, E., Thelin, M. Experimental Prophylaxis of Severe Hemophilia with a Factor VIII Concentrate. *JAMA* 1969, 208(9):1853-1856.
16. Levine, P.H. Hemophilia and Allied Conditions. In: *Current Therapy in Hematology-Oncology*: 1983-1984. Brain, M.C. (ed) New York: BC Decker, 1983, pp. 147-152.
17. Kasper, C.K. Hemophilia and Hemophilioid Disorders. In: Conn, H.F., ed. *Current Therapy*, 4th ed., Philadelphia: Saunders, 1974, pp. 258-263.
18. Kasper, C.K., Dietrich, S.L., Rapaport, S.I. Hemophilia Prophylaxis with Factor VIII Concentrate. *Arch Intern Med* 1970, 125:1004-1009.

Manufactured by:

**GRIFOLS**

Grifols Biologicals Inc.  
Los Angeles, CA 90032, USA  
U.S. License No. 1864

Printed in USA  
Revised January 2004  
©2004

08-8150